UNIVERSITY OF ALBERTA

THE PREVENTION, DIAGNOSIS AND TREATMENT OF REJECTION IN EXPERIMENTAL AND CLINICAL ISLET TRANSPLANTATION

BY

ANDREW MARK JAMES SHAPIRO ©

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment

of the requirements for the degree of

DOCTOR OF PHILOSOPHY

IN

EXPERIMENTAL SURGERY

DEPARTMENT OF SURGERY

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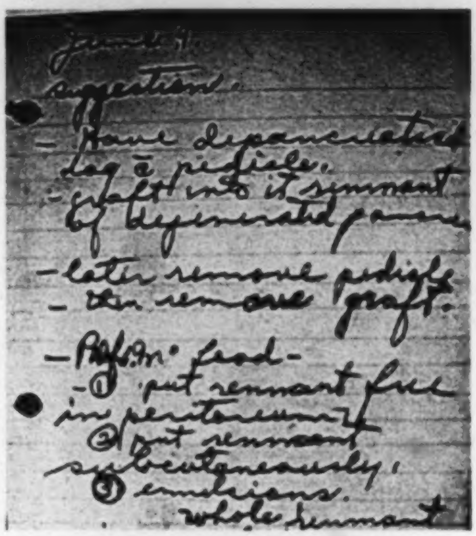
James Shapiro

Dr A M James Shapiro
9120 118th Street
Edmonton, AB

July 9, 2001

June 9th, 1921
Frederick Banting's notebook

"Suggestion. Have depancreatectomised dog… **graft into it remnant** of degenerated pancreas"



June 9.
Suggestion.
- Have depancreat
dog c̄ pedicle.
- graft into it remnant
of degenerated pan
- later remove pedicle
- then remove graft.
- Prof. McLeod -
- ① put remnant free
in peritoneum
② put remnant
subcutaneously.
③ emulsions.
whole remnant

Dr Michael Bliss: "The discovery of insulin"
(with kind permission)

" In general, we may conclude that deplorably little has been undertaken with grafts of the pancreas … much theoretical interest still attaches to transplantation experiments …

Clinically, the method will probably never find application …

Unless some entirely new principle of organ transplantation shall be discovered…"

Dr Frederick Allan, 1913
"Studies concerning glycosuria and diabetes."

UNIVERSITY OF ALBERTA
FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled **THE PREVENTION, DIAGNOSIS AND TREATMENT OF REJECTION IN EXPERIMENTAL AND CLINICAL ISLET TRANSPLANTATION** submitted by **ANDREW MARK JAMES SHAPIRO** in partial fulfillment of the requirements for the degree of **DOCTOR OF PHILOSOPHY IN EXPERIMENTAL SURGERY.**

Dr. Norman M. Kneteman

Dr. Ray V. Rajotte

Dr. John F. Elliott

Dr. Jonathan R.T. Lakey

Dr. Camillo Ricordi

June 28, 2001

DEDICATION

This thesis is dedicated to my wife, Melanie, who has provided me with love, nourishment and support that knows no bounds, and to my children, Emma, Tom, Lara and Harry, whose curiosity and thirst for knowledge has spurred me on.

ABSTRACT

The overriding goal of this thesis was to improve the success of clinical islet transplantation. The overall clinical results of islet transplantation were dismal previously, with fewer than 8% of diabetic patients remaining free of insulin for longer than one year. This failure was attributable to ineffective prophylactic immunosuppression compounded by a lack of tools for early diagnosis of rejection.

An in depth review of the general status of islet transplantation and of current advances in immunosuppression helped to rationalize and design an optimal immunosuppressant strategy to match the specific challenges of an islet allograft.

The canine islet autograft model was used to define harmful diabetogenic interactions of combinations of immunosuppressants, and provided clear evidence that an islet graft cannot tolerate combinations that work for other solid organ grafts – specifically, the combination of glucocorticoids with calcineurin inhibitors caused irreversible damage to islet autograft function.

Studies measuring the concentrations of immunosuppressants in portal and systemic blood in a large animal model characterized the "portal immunosuppressant storm" – a previously poorly characterized phenomenon. These studies demonstrated that an islet graft is highly vulnerable to toxic local immunosuppressant levels after oral therapy, when the graft is exposed to portal blood.

New tools were designed for the early diagnosis of islet rejection. Of three approaches, repeated glucose tolerance tests were the most sensitive and specific at detecting early graft dysfunction preceding graft failure.

Preclinical and clinical data was synthesized in the development of a new "Edmonton Protocol" optimized for islet function. The new protocol was implemented in seven consecutive islet-alone recipients with severe hypoglycemia or who have failed on

intensive insulin therapy. The result delivered unprecedented success in 100% of cases, and was published in the New England Journal of Medicine, and galvanized the clinical islet transplant field.

This work has identified more effective means to prevent, diagnose and treat islet allograft rejection. The combined benefits of more potent but less toxic immunosuppression has led to a dramatic improvement in outcome in clinical islet transplantation, and the "Edmonton Protocol" is currently being further evaluated within the context of an international multicentre trial.

ACKNOWLEDGEMENTS

I wish to express my sincere gratitude to my PhD supervisor, teacher, colleague and friend, Dr Norman Kneteman, for providing an outstanding example of what a surgeon-scientist should be, for his endless enthusiasm, invaluable guidance, encouragement and support, and for providing access to all resources necessary to complete this work.

I am indebted to the members of my supervisory committee – Dr John Elliott, Dr Jonathan Lakey and Dr Ray Rajotte for their constructive criticism and guidance throughout.

For the pre-clinical studies, I acknowledge the dedicated assistance Dr Ergeng Hao, for his technical expertise and dedication to the projects – in a role that extended far beyond that expected of a technician. For the canine islet isolations, I am most indebted to Dr Jonathan Lakey for his expertise, advice and tireless assistance. Dr Diane Finegood provided outstanding collaborative help with computer modeling in the Minimal Model studies, and Dr Tom Churchill provided expert help in regard to biochemical monitoring. I remain extremely grateful to Dr Randall Yatscoff and his team at Isotechnika (Dr Loralie Langman, Heather Gallant and Lorraine Zimmerman) for assistance with immunosuppressant drug level monitoring.

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For the clinical islet transplant studies, I am enormously grateful to a tremendous team of individuals, each of whom has been absolutely critical to the ultimate success of this project. Words are inadequate to describe the contributions of Dr Jonathan Lakey to this project. As an outstanding scientist and Director of the clinical islet isolation laboratory, his contributions to the refinement of protocols for high yield, high viability islet isolation provided a golden opportunity to evaluate new immunosuppressant therapies in the clinic – the dramatic improvements in clinical graft outcome could not have occurred without his support, and that of his isolation team including colleague Dr Ray Rajotte, Dr Tetsuya Kin, head technician Deborah McGhee-Wilson and Richard Wilson, and many other individuals at the SMRI.

Dr Gregory Korbutt as Director of the islet graft characterization laboratory, provided a new insight into functional and immunohistochemical aspects of human islet graft quantification, and has proved to be a wonderful collaborator and colleague.

I have been extremely fortunate to have the opportunity of working alongside Dr Edmond Ryan, who as Medical Director and expert diabetologist provided a unique perspective in appropriate patient selection for islet-alone transplantation. Exchanging insulin for immunosuppression can never be taken lightly, and his expert assistance with all aspects of clinical work-up and long-term follow-up has been enormously appreciated. His research expertise in metabolic monitoring, together with the skills and expertise of Sharleen Imes, have defined new gold standards that are currently being applied to assess islet graft function worldwide. The elegant and informative graft metabolic data contained in Chapter 8 is largely attributable to the input of Dr Ryan and the staff of the clinical islet program, and is a testament to the collaborative nature with which Dr Ryan and I have worked alongside and complemented each other in the research and care of these unique patients. The recent addition of Dr Breay Paty as Fellow in clinical islet transplantation and endocrinology provides the opportunity to take this work to the next stage.

The islet transplant nurse coordinators – Ingrid Larsen, Tracy Davyduke, Cindy Yarmuch, and previously Barbara Waters and Bernadette Hobson, have not only provided superb delivery of care to our islet recipients, but their precise attention to detail, initiative and determination to help build a successful program have all contributed to a professional research team. The additional support of the nurses on 3E3, on the clinical investigation unit and in interventional radiology is much appreciated.

Dr Kevin O'Kelly and his expert colleagues in interventional radiology provided a technical level of expertise in percutaneous portal venous access that has been second to none. Their willingness to make themselves available at any hour of the night or day helped to minimize ischemic injury to the islet grafts, but furthermore provided maximal comfort to our patients by avoiding a need for surgical access.

My clinical colleagues in hepatobiliary, pancreatic and liver transplant surgery, Dr Norman Kneteman and Dr David Bigam provided further support for the clinical islet and research activities by sharing in the peri-transplant patient care, and importantly provided me with an unprecedented level of protected time to concentrate on moving the clinical islet program forward. This opportunity could not have been provided without the absolute support of the Chairman of Surgery, Dr Stuart Hamilton and Dean Lorne Tyrrell, who both recognized the potential value of investing time, funds and expertise in the clinical islet program before any recent positive outcomes occurred.

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The current success of clinical islet transplantation could not have occurred without the generous friendship and strong international collaboration of many individuals over a considerable number of years. I particularly wish to acknowledge the help and support of Dr Camillo Ricordi, whose quest and determination to make progress in islet transplantation goes beyond all personal gain; his willingness to teach, share protocols, methods and equipment in the semi-automated method has been a shining example of the collaborative spirit in which future progress in clinical and experimental islet transplantation will remain secure.

The clinical program could not have moved forward without financial support from many sources. The University of Alberta and Capital Health Authority provided access to all resources at their disposal to allow the program to move ahead. The Alberta Heritage Foundation for Medical Research provided personal funding to me as Clinical Fellow initially, and more recently as Clinical Investigator, and has supported the salaries of my scientist and clinician colleagues involved in this work. The Alberta Foundation for Diabetes Research, through their tireless efforts in raising substantial funds throughout the Province of Alberta, have provided funding for the islet isolation laboratory and have supported clinical nurse coordinator salaries for these studies. The Juvenile Diabetes Foundation International has provided tremendous support both through a JDRF/MRC project grant, and through a Human islet Distribution Grant. Roby and Jennie Roberts have recently provided generous funds that have allowed us to consolidate our clinical team together on one site, and have helped to support staff salaries within the program. This could not have occurred without the dedicated support of Bert Murray and Julie Hamilton from the Faculty of Medicine fundraising office.

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It was only possible to move the clinical program forward within the context of extensive previous background translational research in islet transplantation within the SMRI. The credit for this vision, for his limitless enthusiasm, support and determination not only for the clinical islet program but for my own career as a surgeon-scientist, enormous credit must be given to Dr Ray Rajotte, Director of the Surgical Medical Research Institute, whose life mission has been to advance experimental islet transplantation as a cure for diabetes.

Finally, the biggest credit for the success of the clinical islet program must go to our 18 consecutive islet recipients, and to many more Canadian patients on our active waiting lists. These brave individuals placed their faith, trust and respect in our clinical team when we re-opened the clinical islet program; we could not promise a successful outcome, and could only estimate the potential risks that each of these individuals might face. Their willingness to submit to an untried and untested clinical protocol, and unquestioned belief in us as clinicians and scientists, allowed us to move forward.

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LIST OF ABBREVIATION

AGE	Advanced glycation end-products
PKC	Protein kinase C
RAGE	Receptor for advanced glycation end products
PERV	Pig endogenous retrovirus
CsA	Cyclosporine
NFAT	Nuclear factor of activated T-cells
mTOR	mammalian target of rapamycin
PTLD	Post transplant lymphoproliferative disorder
K_G	glucose decay constant
GAD_{65}	Glutamic acid decarboxylase
b-gal	beta-galactosidase
WF	Wistar-Furth strain
Lew	Lewis strain
HBSS	Hank's balanced salt solution
S_{AUC}	Stimulated area-under-the-curve
SI	Stimulation Index
DMEM	Dulbecco's modified Eagle's Medium
FCS	Fetal calf serum
RLU	Relative light units
Stationary	modified stationary digestion
Chopped	chopped tissue digestion
PSD	penicillin, streptomycin, dextrose
IVGTT	intravenous glucose tolerance test
SAUC	stimulated area under the curve
HPLC	High-pressure liquid chromatography
AUC_{4h}	4-hour Area under the curve
AUC_{12h}	12-hour Area under the curve
C_{max}	Maximal concentration
t_{max}	Time to maximal concentration
FSIGT	Frequently sampled intravenous glucose tolerance test
MINMOD	Minimal model analysis
FPIA	fluorescence polarization immunoassay
AIRg	Acute insulin response to glucose

PREFACE

Clinical islet transplantation offers the promise of cure in Type 1 diabetes mellitus by providing lasting freedom from insulin injection, glucose monitoring and dietary restriction. Furthermore, it offers the potential to prevent, stabilize or even reverse secondary diabetic complications when applied sufficiently early in the course of the disease. This promise has been difficult to sustain however in previous worldwide trials, where in over 447 clinical islet transplants performed as reported to the Islet Transplant Registry, less than 8% of patients remained free of insulin beyond one year.(*1, 2*)

Despite these dismal results overall, more recent data suggests that results are steadily improving. The best results obtained under cyclosporine + glucocorticoid-based immunosuppression were reported by the Giessen, Geneva (GRAGIL) and Milan groups, with 20% insulin independence and 50% C-peptide positive rates by one year (unpublished data). This limited success in clinical trials contrasts markedly with the dramatic and durable success seen in animal models of islet transplantation, where since the early 1970s islet transplantation was found to routinely cure diabetes in mice and rats(*3, 4*).

The **central hypothesis** of this thesis is that the disparate results seen between clinical and laboratory, and between islet and other forms of clinical solid organ transplantation, are a **direct result of inappropriate and ineffective prophylactic immunosuppression**. High dose cyclosporine + glucocorticoid therapy is directly toxic to beta cells, promotes insulin resistance, and **fails to prevent acute rejection or autoimmune recurrence** of diabetes. These challenges are compounded by an **inability to diagnose and control early islet rejection**.

The corollary is that more potent and less toxic immunosuppression, tailored to meet the specific needs of islet transplantation, coupled with an effective early diagnostic marker of islet rejection, could have major impact in enhancing the durability of insulin independence after clinical islet transplantation to match the success enjoyed by most other organ transplants.

CHAPTER 1:

GENERAL INTRODUCTION TO CURRENT CHALLENGES IN DIABETES, REVIEW OF ALTERNATIVE THERAPEUTIC APPROACHES, WITH EMPHASIS ON THE CURRENT AND FUTURE POTENTIAL OF CLINICAL ISLET TRANSPLANTATION

1.1 INCIDENCE, CLASSIFICATION, ETIOLOGY AND PATHOGENESIS OF DIABETES

Diabetes mellitus is a clinical syndrome of disordered carbohydrate, lipid and protein metabolism defined by the presence of hyperglycemia. The syndrome was first recognized by ancient Egyptian physicians and inscribed in the Ebers Papyrus in 1500 BC, which was discovered later in a tomb in El Assassif(*1*). Aretaeus of Cappadocia (second century AD) applied the term "*diabetes*" from the Greek for "*flow through a siphon*," and described "*the moist and cold wasting of the flesh and limbs into urine*(*2*)." Hindu physicians in the eighteenth century discovered the "*honeyed*" taste of the urine of diabetic patients, and added the term "*mellitus*."

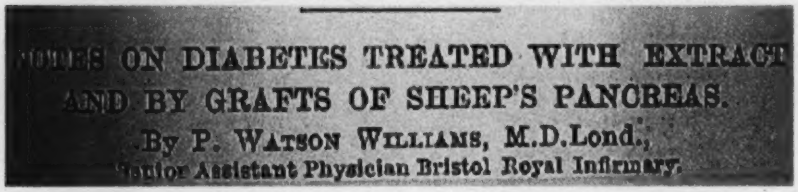
An estimated 130 million people have diabetes worldwide. It affects 6% of the population of developed countries including North America, being the third commonest disease, and fourth leading cause of death(*3*). Of the eight million patients diagnosed with diabetes in North America, one million are Type 1 and seven million are Type 2; a further estimated eight million have Type 2 diabetes but have not yet been diagnosed. There are at least 30,000 new cases of Type 1 diabetes diagnosed per year in North America, and the incidence is rising annually(*4*). In Canada, an estimated 1.5 million people have diabetes at the current time, and this is expected to reach 3.0 million by the year 2010(*5, 6*). A doubling in world incidence is predicted, with a projected total exceeding 300 million people within the next 25 years(*7*). The increase will occur mainly in type 2 diabetes, which unfortunately can no longer be regarded as "adult-onset" since there are now over 5 million children diagnosed in North America(*8*). The incidence of type 1 diabetes (autoimmune) is also rising annually, with a current incidence of 30,000 new cases per year in North America(*4, 9*). A prospective longitudinal study from Finland demonstrated a 2.4% annual increase in incidence of Type 1 diabetes in children, and in the United Kingdom the reported incidence of Type 1 diabetes rose from 4.2 to 9.9 per 100,000 per year between 1973 and 1988(*10-12*). In Edmonton, Alberta, Toth et al found an age-adjusted incidence of Type 1 diabetes of 25.7 per 100,000 per year over a six-year period in children of European ancestry aged 10 to 14 years, occurring mainly in the winter months, representing one of the highest Type 1 diabetes rates in North America(*13*). The societal cost estimates for diabetes and its inevitable sequelae are enormous, consuming between 9-15% of healthcare expenditure in developed countries(*14*). This amounted to $98 billion in the USA, approximately $9 billion in Canada, and £4.9 billion (or £558,379 per hour) in the UK in the year 2000.

Diabetes mellitus was traditionally classified by the extent a patient was dependent on insulin (Type 1 – insulin dependent vs. Type 2 – insulin resistant)(*15*). However, the World Health Organization and American Diabetes Association classifications are currently in flux to a system that defines diabetes in terms of pathogenesis, acknowledging the heterogeneity of processes leading to the disease(*16*). The four main sub-groups are: Type 1 (immune mediated or idiopathic beta-cell dysfunction leading to absolute insulin deficiency); Type 2 (adult onset, which may originate from insulin resistance and relative insulin deficiency or from a secretory defect); Type 3 (covering various defined genetic defects of beta-cell function, insulin action and disease of the exocrine pancreas); and Type 4 (gestational diabetes)(*17*). Furthermore, a change in diagnostic threshold of fasting glucose concentration, from 7.8 mmol/l to 7.0 mmol/l has been proposed, with introduction of new intermediate category of "impaired fasting glucose" (range ≥ 6.1 but ≤ 7.0 mmol/l). A random plasma glucose of ≥ 11.1 mmol/l, confirmed by a fasting glucose ≥ 7.0 mmol/l on the subsequent day defines the current diagnosis of diabetes in a patient with classical symptoms, and this altered definition has not affected the true prevalence of diabetes to date(*18*).

The etiology of type 1 diabetes is multi-factorial and incompletely understood. Genetic susceptibility clearly enhances risk of autoimmune beta-cell damage by viral or environmental toxins. The lifetime risk of type 1 diabetes in a first-degree relative is 6%, but this rises to 25 - 50% in identical twins(*19*). Where a first-degree relative has high-titer islet cell antibodies, the risk increases substantially to 8% per year, with an accumulated risk of 70% within 10 years(*20, 21*). Genetic susceptibility accounts for up to 40% of risk of Type 1 diabetes, and is predominantly associated with an MHC HLA locus on the short arm of chromosome 6 (IDDM1 locus) or the IDDM2 locus (insulin gene and flanking regions on chromosome 11p15), but at least 13 other loci have been identified to date(*22-26*). Early consumption of cow's milk proteins may play a role in initiation, related to antibody cross-reactivity between beta-casein and several beta-cell islet proteins(*27, 28*).

Inheritance and expression of type 2 diabetes is also multi-factorial and dependent on multiple genes interacting with each other in addition to environmental, lifestyle, and dietary factors. The recent discovery of a strong association between type 2 diabetes and a series of polymorphisms in the Calpain 10 gene appear to explain the increased susceptibility for type 2 diabetes in high-risk populations(*29*). The gene encodes for cysteine protease, an enzyme involved in splicing or degrading of proteins. Calpain 10 polymorphisms could soon be used as marker genes in at-risk populations. An additional gene encoding tyrosine phosphatase 1B (PTP-1B) has been implicated in regulation of insulin resistance in Type 2 diabetes through its effects on the insulin receptor(*30*). This may pave the way for innovative therapies in type 2 diabetes, and may allow improvement in graft function after islet transplantation, particularly in the setting of a sub-therapeutic islet engraftment mass.

1.2 HISTORICAL PERSPECTIVE

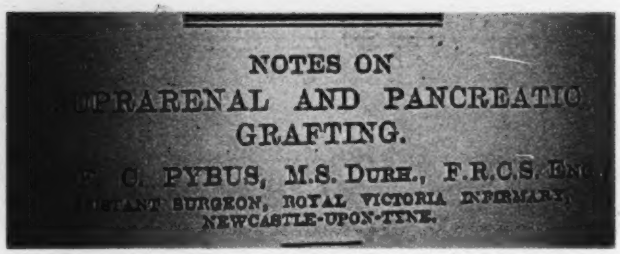


NOTES ON DIABETES TREATED WITH EXTRACT
AND BY GRAFTS OF SHEEP'S PANCREAS.
By P. WATSON WILLIAMS, M.D.Lond.,
Junior Assistant Physician Bristol Royal Infirmary.

The first clinical attempt at islet/pancreas transplantation in the treatment of diabetes occurred on December 20th 1893, 28 years before the discovery of insulin(*31*). Dr Watson-Williams and his surgical colleague Mr. Harsant, working at the Bristol Royal Infirmary in England UK, transplanted three pieces of freshly slaughtered sheep's pancreas, "*each the size of a Brazil nut*," into the subcutaneous tissues of a 15 year old boy dying from uncontrolled ketoacidosis. The operation, performed under chloroform anesthesia, was completed "*within twenty minutes of the death of the sheep*." Although there was temporary improvement in glucose before death three days later, this xenograft was destined to fail without immunosuppression.

Figure 1.1: Oscar Minkowski – discovered link between pancreas and diabetes (1892)

The idea was not new, for Oscar Minkowski had already carried out a similar procedure in a pancreatectomized dog in the previous year (1892), and had described a temporary reduction in glycosuria(*32*). These experiments were published just three years after Joseph von Mering and Oscar Minkowski had made the monumental discovery that the pancreas was linked to diabetes by surgical removal of a dog's pancreas with onset of polyuria and glycosuria(*33*).



NOTES ON
UPRARENAL AND PANCREATIC
GRAFTING.
C. PYBUS, M.S. DURH., F.R.C.S. EN
STANT SURGEON, ROYAL VICTORIA INFIRMARY,
NEWCASTLE-UPON-TYNE.

In July 1916, surgeon Frederick Pybus carried out further pioneering clinical attempts by subcutaneous human cadaveric pancreatic fragment transplantation in Newcastle-on-Tyne, England UK(*34*). One of his two patients was reported to show temporary reduction in glycosuria.

Four years later, on October 31st 1920, the idea occurred to Frederick Banting that ligation of the pancreatic duct in dogs might lead to acinar degeneration and enhanced recovery of the "*internal secretions*" for treatment of diabetes(*35*). The effect was dramatic, and ongoing studies by Banting, Best, Collip and Macleod rapidly led to the introduction of exogenous insulin into clinical practice in 1922. By the following year Eli Lilly and Company was producing insulin in virtually unlimited quantities for the widespread treatment of diabetics(*36*). Diabetes was transformed from being a rapidly fatal death sentence after onset of ketoacidosis to a chronic incurable illness with most diabetics developing one or more end-stage secondary complications during their lifetime.

1.3 PRIMARY AND SECONDARY PREVENTION OF DIABETES

The ultimate goal in diabetes disease control is primary and secondary prevention by appropriate early intervention in an at-risk population, and over the past decade this has been a major focus of diabetes research. Most strategies have been aimed at the newly diagnosed Type 1 diabetic or at-risk pre-diabetic where early immune activation has already occurred. A Canadian-European randomized trial addressing the effects of systemic immunosuppression with cyclosporine provided initial optimism that non-insulin requiring remission could be induced in a proportion of diabetics with early disease(*37*). Enthusiasm waned rapidly as data emerged showing that this intervention only delayed inevitable disease progression, and furthermore any protective effects were overshadowed by cyclosporine-induced accelerated nephrotoxicity in renal biopsies at one year(*38*). Other strategies have included early initiation of subcutaneous insulin (for islet rest or parenteral antigen effect), vitamin B_3, nicotinamide, or vaccination by a variety of immunomodulatory adjuvants or auto-antigens, with limited promise to date(*39-42*).

Most recently, Bluestone and colleagues developed a humanized FcR non-binding anti-CD3 mAb (hOKT3γ_1-Ala-Ala) that targets only activated T-cells, and lacks toxicity previously associated with conventional anti-CD3 mAb therapy. The mAb was genetically engineered from murine OKT3 mAb by grafting the six complementarity-determining regions within a human IgG1 mAb, and where the C_H2 region was altered by site-directed mutagenesis to eliminate T-cell activation(*43*, *44*). FcR non-binding anti-CD3 Abs have short-lived effects on naïve T-cells but deliver a partial signal in activated T-cells resulting in clonal inactivation of Th1 cells while sparing the suppressive Th2 T cell subset. This approach was developed to selectively inhibit and tolerize the inflammatory subset of auto- and allo-reactive Th1 cells, thus preventing inflammation and restoring peripheral self-tolerance. Short-term immunotherapy of overtly diabetic NOD mice with an anti-CD3 mAb restored durable self-tolerance to autoantigens and prevented autoimmune recurrence in syngeneic islet grafts – this ability to restore self-tolerance in the presence of an ongoing autoimmune response is unprecedented(*45*). A 5-consecutive-day treatment induced a complete and durable remission beyond 8 months in follow-up, and subsequent syngeneic islet grafts were not rejected(*46*). Preliminary data from a type 1 diabetes primary prevention trial in patients treated with hOKT3γ_1Ala-Ala within six weeks of diagnosis, suggest that marked improvement in Phase 1 insulin release can be restored. Twenty-one patients have received therapy in this trial to date, with 12 patients followed beyond one year. 8/12 patients demonstrated more C-peptide production than was evident at time zero, compared to 2/10 in the control group, and this was associated with a 40% reduction in HbA1C at 6 months, compared with pre-treatment controls (J Bluestone, personal communication). Side effects have been mild, including mild self-limiting rash, anti-idiotypic antibody response in one third of cases.

Regarding the primary prevention of Type 2 diabetes, there is a surprising lack of randomized clinical trial data to prove conclusively that modification of body weight, body fat distribution, physical exercise or diet can modify the risk of diabetes(*47*). This may in part be due to the fact that modification of such factors is ineffective in the long term for patient cohorts with Type 2 diabetes.

1.3.1 SECONDARY DIABETIC COMPLICATIONS – INCIDENCE, ETIOLOGY & CONTROL

While the discovery of insulin radically transformed the short-term outlook of Type 1 diabetics worldwide, it failed to prevent the inexorable progression of one or more secondary diabetic microvascular and macrovascular complications in most patients (see table 1), leading to a shortening in lifespan of at least ten years compared to age-matched non-diabetic controls(*48*). Diabetes mellitus is currently the leading cause of renal failure, blindness, amputation and impotence in adults, and accounts for in excess of 160,000 deaths per year in the US. Most deaths relate to the increased morbidity in patients with renal failure and cardiovascular disease in a subgroup of 35% of patients who develop diabetic nephropathy(*49*). A large 10 year cohort study in Denmark demonstrated that increased urinary albumin excretion, poor glycemic control and short stature were all independent risk factors for elevated mortality in diabetics overall, and that the presence of microalbuminuria or overt nephropathy, arterial hypertension, smoking and age were predictive of excess cardiovascular mortality in diabetics(*50*). Interestingly, a 1% increase in glycosylated hemoglobin A_1C was associated with an 11% increase in risk of death in this trial.

It has become increasingly apparent that the degree of secondary diabetic complications in an individual is strongly related to extended exposure to hyperglycemia(*51-54*). Chronic elevation in blood glucose results in glycation of extracellular matrix proteins by non-enzymatic conjugation with glucose, leading to formation of advanced glycation end-products (AGE). In turn this leads to increased oxidative stress, enhanced collagen cross-linking, capillary closure, accelerated atherosclerosis and ultimately end-organ ischemia(*55*).

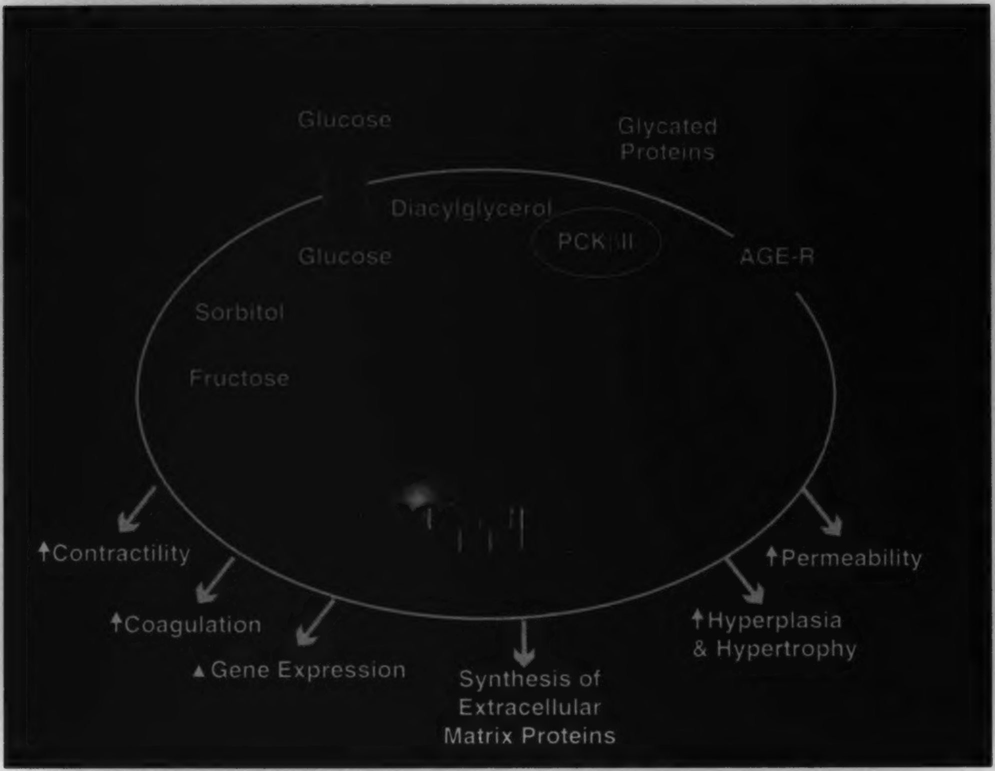

Glucose
Glycated Proteins
Diacylglycerol
PCK II
Glucose
AGE-R
Sorbitol
Fructose
↑Contractility
↑Coagulation
▲Gene Expression
Synthesis of Extracellular Matrix Proteins
↑Hyperplasia & Hypertrophy
↑Permeability

Figure 1.2: Biochemical pathways involved with end-organ injury in hyperglycemia
(adapted from Feener et al., Vascular dysfunction in diabetes mellitus. Lancet 1997; 350 (suppl 1): 9-13)

AGE receptors have been identified on the cell surface that bind glycated proteins, increasing *de novo* synthesis of diacylglycerol and activity of protein kinase C (PKC), with increased protein phosphorylation and eventual tissue injury. A second pathway involves sorbitol accumulation via aldose reductase, altered intracellular redox and oncotic balance, and has been implicated in the formation of cataracts(*56*).These pathways have been integrated and summarized in an excellent review by Feener et al(*52*).

The discovery and subsequent cloning of the receptor for advanced glycation end-products (RAGE) has provided a unique opportunity to prevent secondary diabetic complications through interference with these pathways(*57-59*). The development of RAGE ant-sense peptides now provides a unique opportunity to prevent progression of microangiopathy in small animal models, and ultimately if safe and effective, preliminary clinical trials should be forthcoming. Differential expression of the RAGE receptor may partly explain why some individuals have accelerated secondary complications whereas others are protected despite sub-optimal glycemic control(*60*). Validation of RAGE markers in the diabetic population might one day provide an effective screening tool to predict which patients with Type 1 diabetes would best benefit from early or even pre-emptive islet transplantation.

The landmark multicentre trial, the Diabetes Control and Complications Trial (DCCT), in which patients were randomized to conventional twice daily subcutaneous insulin injections or to more intensive therapy, has clearly shown the clinical relevance of the above complex pathway interactions, and proved that intensified glycemic control with correction in glycosylated HbA_1C, can significantly decrease microalbuminuria by 35%, clinical neuropathy by 60% and retinopathy by 34% to 76%(*61*). The penalty for tight glycemic control from intensified exogenous insulin was a three times increase in serious hypoglycemic events (including recurrent seizure or coma) compared to the control arm(*62*).

Despite optimization of intensive subcutaneous insulin delivery, this approach will never provide perfect physiological pulsatile matching for moment-to-moment variability in serum glucose, and is associated with peripheral hyperinsulinemia, which may be partially implicated, in accelerated atherogenesis in diabetes(*63, 64*). It is evident that alternative approaches to subcutaneous insulin must be sought if the long-term quality of life and risk of secondary morbidity are to be minimized for patients with Type 1 diabetes.

1.4 ALTERNATIVE THERAPEUTIC STRATEGIES FOR ENDOCRINE REPLACEMENT IN THE TYPE 1 DIABETIC

Recent progress has occurred in a variety of strategies aimed at endocrine replacement for the Type 1 diabetic. These include whole pancreas transplantation, the artificial pancreas and islet transplantation therapies, and are reviewed below.

1.4.1 PANCREAS TRANSPLANTATION

Dramatic improvement in outcome has occurred in clinical vascularized pancreas transplantation since the first procedure was carried out by Kelly and Lillehei at the University of Minnesota in 1966(*65*). The earliest attempts met with dismal mortality rates in excess of 60% and graft survival of only 3% at one year, related to uncontrolled sepsis from failure of duodenal anastomotic healing in the face of high dose steroids(*66, 67*). In 1983 two crucial developments immediately enhanced the success of the procedure – first the introduction of cyclosporine provided enhanced immunologic potency and reduced sepsis with better tissue healing through its steroid sparing potential; secondly, both Cory and Sollinger described techniques for bladder drainage of pancreatic exocrine secretions, which provided better immune monitoring through urinary amylase assessment, a lower anastomotic leak rate with reduced gram negative sepsis(*68-70*). Subsequent improvement in outcome led to endorsement of simultaneous pancreas-kidney transplantation as recommended treatment of the Type 1 diabetic presenting in non-reversible renal failure(*71*).

Pancreas transplantation is currently the only treatment of Type 1 diabetes that consistently restores sustained endogenous secretion of insulin responsive to normal feedback control, leading to complete normalization of glycosylated HbA$_1$C, which far surpasses that achieved in the DCCT trial(*72, 73*).

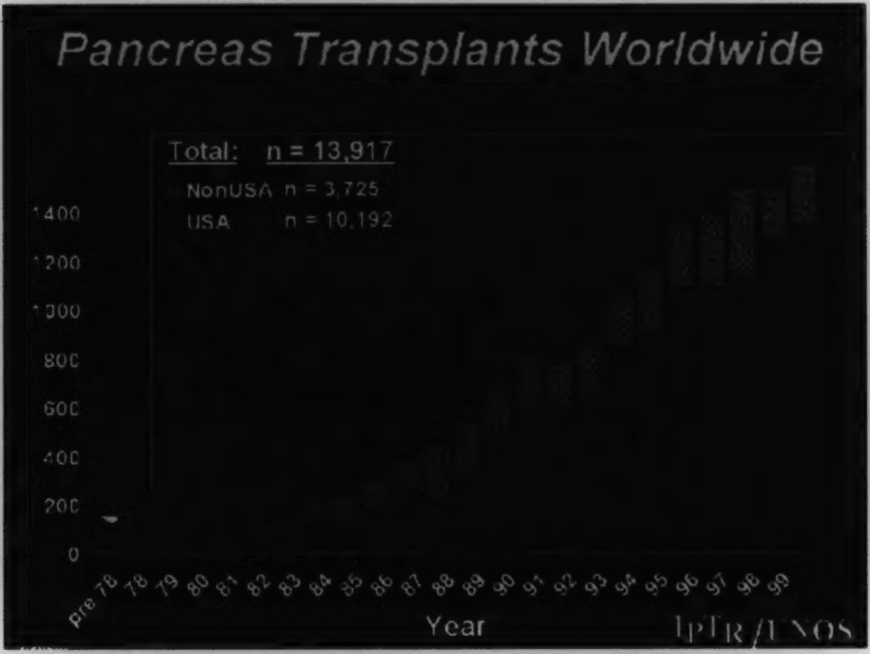


Figure 1.3: Increasing activity in whole pancreas transplantation
(with permission from the Pancreas Transplant Registry)

As of December 2000, over 13,917 pancreas transplants have been performed worldwide with an annual rate exceeding 1500, as reported to the International Pancreas Transplant Registry(*74*).

The majority of transplants have been simultaneous pancreas-kidney, although an increasing number of solitary pancreas transplants are now being performed. For simultaneous pancreas-kidney transplants, the actuarial survival of patients and of functional pancreas grafts (with complete insulin independence) are currently 94% and 89% at one year and 81% and 67% at five years respectively, according to registry data(*74*).

The results of pancreas-alone grafts remain inferior to simultaneous pancreas-kidney grafts, according to the Registry data. However, pancreas alone transplantation can lead to excellent outcomes in carefully selected individuals under tacrolimus-based immunosuppression, with one-year graft survival is 80% to 90%, with corresponding patient survival as high as 95%(*75-77*).

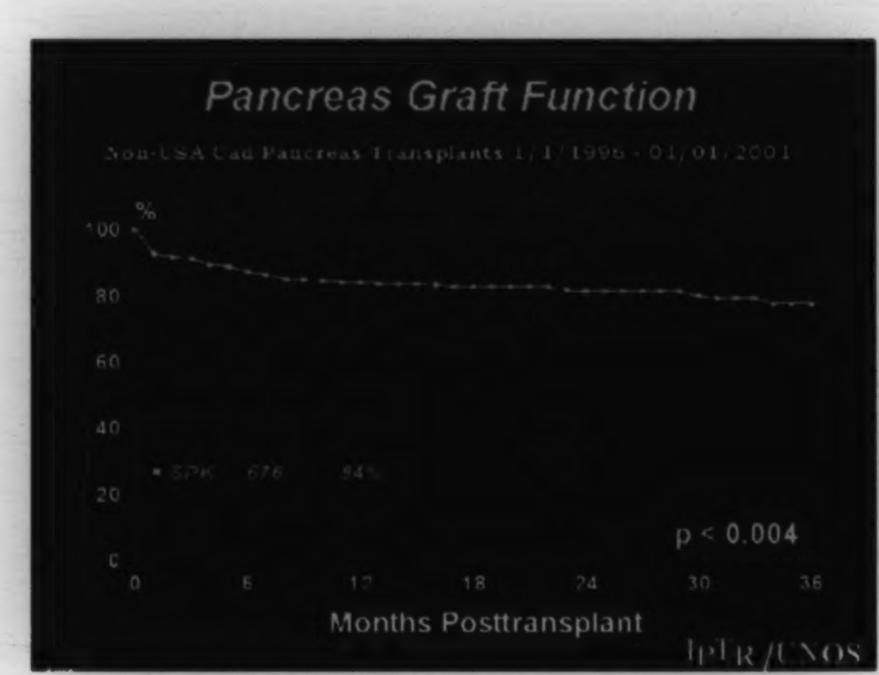


Figure 1.4: Pancreas graft function is inferior for pancreas alone grafts
(with permission from the Pancreas Transplant Registry)

Patient survival improves by at least 10% by five years and by up to 59% at ten years following combined transplantation compared with kidney-alone transplantation in the Type 1 diabetic(*78, 79*). Freedom from insulin, blood glucose monitoring and dietary restriction improves the overall quality of life for the diabetic undergoing successful pancreas-kidney transplantation, but scores generally fail to match those of the general non-diabetic healthy population by one year post-transplant(*80-82*). Quality of life improvement is particularly evident in patients with hypoglycemic unawareness, brittle diabetes or gastroparesis(*83*).

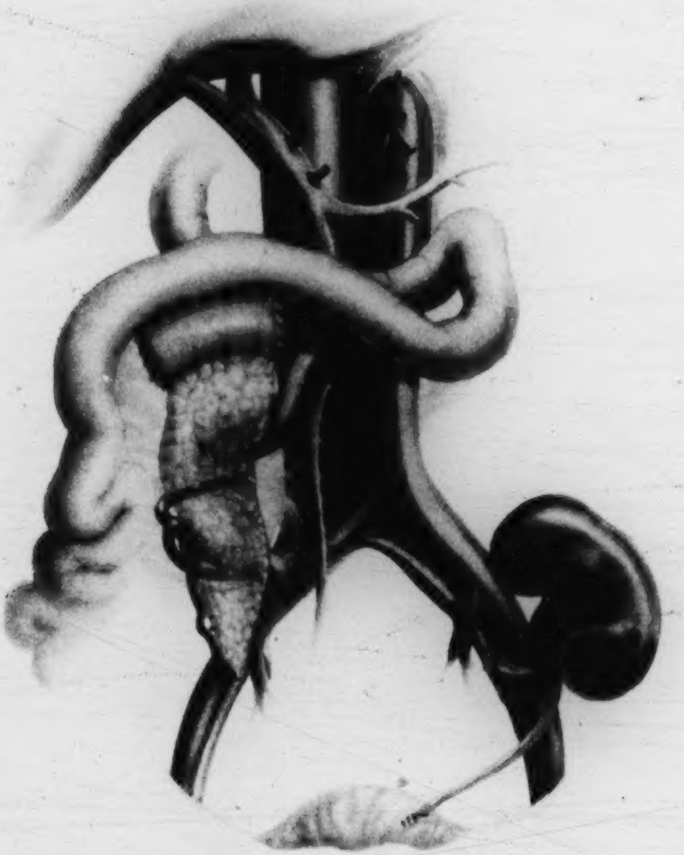


Figure 1.5: Portal-enteric pancreas grafts have less rejection and improved outcome

(with kind permission – Dr Stephen Bartlett)

Recent advances in surgical technique, immunosuppression and post-transplant monitoring have had major impact in reducing the morbidity of patients undergoing simultaneous pancreas-kidney and solitary pancreas transplantation. A recent return to enteric exocrine drainage by graft duodenojejunal anastomosis has dramatically reduced complications of urinary tract infection, urethritis, urethral stricture and metabolic acidosis, and therefore the need to perform enteric conversion in up to 33% of cases(*84-87*).

Primary portal venous graft drainage avoids the insulin resistant effects of peripheral hyperinsulinemia(*88*), may improve metabolic control(*89-92*), has been associated with reduced incidence of acute rejection in at least one clinical study (perhaps through direct portal antigen delivery)(*93, 94*), and is often technically less challenging to perform than systemic drainage(*95*).

It remains to be seen whether these benefits translate to improvement in outcome in prospective trial, however. Marked reduction in acute rejection incidence with combined tacrolimus and mycophenolate, monitoring of serum amylase and lipase with ultrasound-directed percutaneous pancreatic biopsy where indicated, have compensated for the loss of urinary amylase as a marker of pancreatic rejection in enterically drained pancreas transplants(*75, 96*).

The impact of pancreas transplantation in control of progression of secondary complications has been more challenging to prove, largely because the procedure has been applied late in the course of Type 1 diabetes and typically after more than 20 years of disease(*97*). When diabetic patients undergo kidney transplantation alone, histological changes of early diabetic nephropathy usually recur in the graft within two years, and progress to end-stage disease after 10 years(*98*). Combined pancreas-kidney transplantation is protective to the kidney, prevents recurrence of diabetic nephropathy, and extends the projected half-life of the kidney transplant from 7.9 to 11.3 years(*73*). Furthermore, Fioretto et al have shown that solitary pancreas transplantation will reverse lesions of early diabetic nephropathy in non-uremic Type 1 diabetics, but significant reversal did not occur after 5 years, but only after 10 years of perfect metabolic control(*99*).

Several recent studies have shown that progression of peripheral and autonomic neuropathy not only halts but also may be completely reversed by successful pancreas transplantation, even if polyneuropathy is advanced(*100, 101*). The most impressive improvements in neuropathy have been in recovery of gastroparesis, and restoration of hypoglycemic awareness and response to epinephrine(*83, 102*). The course of diabetic retinopathy seems less favorably influenced by pancreas transplantation, likely because proliferative change and macular scarring has already occurred. Advanced retinopathy fails to remit, but early retinopathy followed for longer than four years is stabilized but does not reverse after pancreas transplantation(*103-105*). Longer-term prospective studies are required to establish the impact of pancreas transplantation on atherogenesis, cerebrovascular disease and cardiovascular mortality, when applied earlier in the course of disease.

The above benefits of pancreas transplantation come with a cost of potential increased morbidity related to surgery and post-transplant immunosuppression, and may place additional financial burden on the health-care system(*106*). Although technical complications have diminished with evolution in surgical technique and improved immunosuppression, the morbidity of rejection, graft pancreatitis, anastomotic leak or vascular thrombosis can occasionally lead to prolongation in hospital stay and increased mortality. The risk-benefit ratio of pancreas transplantation must be individualized to take account of the patient's long-term risk of morbidity and early death from inadequately controlled diabetes; the risk may be readily justified in carefully selected patients undergoing combined pancreas-kidney transplantation, but the balance of indications in pancreas-alone transplantation remains more controversial at the present time(*72, 79, 107-109*).

1.4.2 THE ARTIFICIAL PANCREAS

Data from the Diabetes Control and Complications Trial (DCCT) demonstrated unequivocally that intensive control of blood glucose, with correction of glycosylated hemoglobin A_1C, will delay progression of secondary diabetic complications(*110*). Intensive therapy in the DCCT was achieved by multiple insulin injections of three or more times per day, or by use of a continuous external subcutaneous insulin pump. The natural extension of this approach is to provide an implantable artificial pancreas to deliver insulin in a controlled near-physiological manner. Most systems are based on a closed feedback loop response to dynamic glucose sensing, an insulin pump and a control system. The attraction of this system is that it does not require systemic immunosuppression, and is not limited by a finite supply of donor organs. One of the major limitations has been in achievement of a long-term stable glucose sensing system. Enzymatic glucose sensors were originally developed in the early 1960s, and initially applied in the brewing industry, but have not been used successfully on a long-term basis in humans(*111, 112*). Most utilize glucose oxidase laminated and immobilized on a membrane, with generation of hydrogen peroxide either measured directly or indirectly by oxygen consumption(*113*). Bio-compatibility remains a major issue, with foreign body inflammatory response interfering with sensor function, necessitating frequent re-calibration(*114*).

Subcutaneous monitoring via needle electrodes is inherently associated with a lag in sensor responsiveness, limiting the dynamic feedback in a closed loop system. Sternberg et al found a delayed response time of approximately 12 minutes in subcutaneous sensed glucose compared with blood glucose in human subjects(*115*). Sampling of dermal interstitial fluid for glucose monitoring is another approach that has shown promise, but is also limited by lag feedback delay(*116*). Intravascular enzymatic glucose sensing has not been applied long-term in humans to date because of concerns of increased infection, bleeding or thrombotic risk, although similar probes have been used successfully for more than 100 days in dog models(*117*).

Recent progress has been made in the use of optical glucose sensor technology, where the absorption pattern of a near-infrared light (700-1300nm) may correlate with glucose concentration(*118*). However the absorption of hemoglobin and other serum proteins in this waveband may limit the sensitivity of this approach, necessitating complex mathematics regression modeling to compensate(*119*). Integration of multiple sensor signal arrays tuned to slightly different wavelengths in a system known as kromoscopy may provide enhanced sensitivity, but this has not been tested clinically at present(*120*).

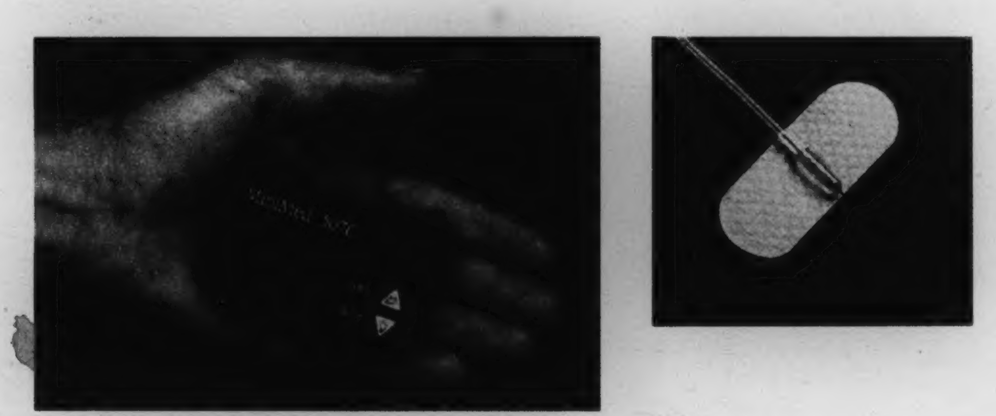


Figure 1.6: Miniaturized external insulin pump

Impressive progress has been made in the miniaturization and implantability of insulin pump and control systems in recent years. The large bedside devices developed in the 1970s have been transformed into external pumps of similar size to a pager, which may be worn comfortably. One of the difficulties with these devices is that they may become dislodged readily during physical exercise, or the access site may become infected on the skin surface.

Implantable pumps may deliver insulin intravenously or directly into the peritoneal cavity via a fine catheter. While subcutaneous and intravenous insulin delivery is associated with marked peripheral hyperinsulinemia, intraperitoneal insulin release provides rapid and predictable absorption into the portal system, resulting in physiological hepatic delivery and more optimal glycemic control(*121-123*). In a clinical cross-over trial comparing intensive subcutaneous insulin with programmable implanted insulin pumps in 56 patients, pumps functioned for periods up to 1.7 years, and the most impressive finding was a marked reduction in incidence of severe hypoglycemia when compared with intensive subcutaneous insulin(*124*).

Current implantable insulin pumps are the size of a cardiac pacemaker and provide intraperitoneal pulsatile insulin delivery coupled with a hand-held programming device. They typically require refilling every three months by percutaneous port inoculation, and have been implanted in over 600 patients worldwide to date with no major safety concerns. The utility of these devices will be enhanced enormously once a satisfactory solution to closed loop glucose sensing is realized.

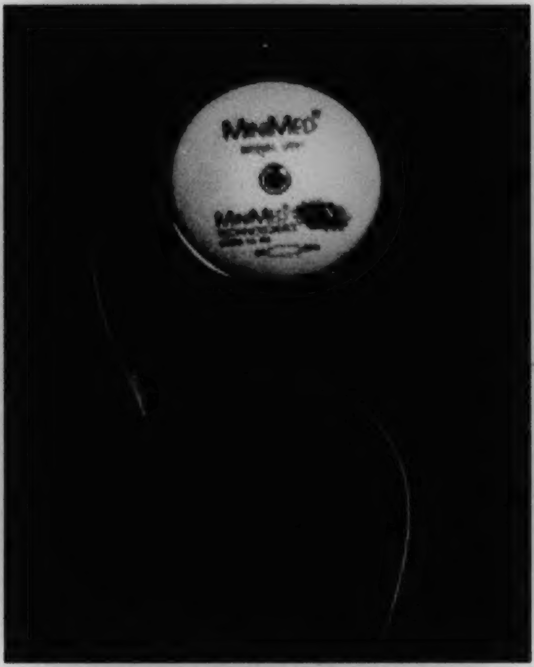


Figure 1.7: Implantable insulin pump

1.5 ISLET TRANSPLANTATION

1.5.1 EXPERIMENTAL ISLET ISOLATION AND TRANSPLANTATION

The average adult human pancreas weighs 70 grams, contains an average of 1 to 2 million islets (although Robertson *et al* quote a maximal upper limit of 14.8 million islets) of mean diameter 157 µm, constituting between 0.8 – 3.8% of the total mass of the gland(*125, 126*). The successful isolation of large numbers of viable islets has proven to be a challenge, however. The era of experimental islet research began in 1911 when Bensley stained islets within the guinea pig pancreas using a number of dyes, and was able to pick free the occasional islet for morphological study(*127*). Armed with watch-maker's forceps, hypodermic needles and a binocular microscope Hellerström developed methods in 1964 for free-hand micro-dissection of small numbers of islets for biochemical and physiological study(*128*). These techniques were effective in an obese hyperglycemic strain of mouse with uniquely large islets, but were impractical in most other species. Prompted by a need for large-scale isolation to further *in vitro* studies, Moskalewski introduced a mechanical and enzymatic method of dispersion of pancreatic tissue in 1965 using bacterial collagenase derived from *Clostridium histolyticum*(*129*). Although the collagenase destroyed many islets, it did permit complete separation of islets from surrounding acinar tissue, with demonstrable viability in culture and appropriate b-cell degranulation in hyperglycemic challenge. Lacy and Kostianovsky introduced two further modifications in 1967 that considerably improved islet yield and recovery(*130*): Mechanical disruption of the pancreas by ductal injection of a balanced salt solution greatly increased the subsequent penetration of collagenase, with consequential enhanced islet release. They further discovered that islets could be separated from digested acinar tissue by differential density elutriation on discontinuous sucrose gradients, but these islets failed to release insulin in response to a glucose challenge which was presumed to be the result of hyperosmolar sucrose injury from cellular dehydration and islet exhaustion. Lindall *et al* found that replacement of sucrose gradients with Ficoll, a high molecular weight polymer of sucrose, led to more efficient islet separation, and Scharp and colleagues further showed that dialyzed Ficoll provided islets that responded appropriately *in vitro*(*131, 132*).

These preliminary studies paved the way for transplantation studies in diabetic rodents. Younoszai *et al* in 1970 were the first to demonstrate amelioration of the diabetic state in rats by intraperitoneal implantation of allografted islets, with improvement in glycuria but only temporary improvement in glycemia(*133*). Two years later, Ballinger and Lacy showed sustained improvement (but not complete correction) of chemical diabetes in rats receiving 400 – 600 islets delivered intraperitoneally or intramuscularly, with graft excision inducing return of diabetes(*134*). It was not until Rechard and Barker transplanted larger numbers of islets (800 – 1200) into the peritoneal cavity in 1973 that chemically induced diabetes was effectively cured for the first time(*135*).

Searching for optimal sites for islet implantation, Kemp *et al* found that intra-portal embolization of only 400 – 600 rodent islets to an intra-hepatic site resulted in complete reversal of diabetes within 24 hours, whereas a similar islet load placed intraperitoneally or subcutaneously was inadequate(*136*). Portal embolization was thus recognized to be the most efficient site for islet implantation in the rodent, with the benefit of high vascularity, proximity to islet-specific nutrient factors and physiological first-pass insulin delivery to the liver. It has recently become apparent that once embolized to the liver, islets undergo a process of angiogenesis and neovascularization to form a microvascular network and to re-establish nutritional blood supply. In the mouse, capillary sprouts and arterioles arise within 2 to 4 days, interconnect by day 6, and the process is completed by day 10 to 14(*137*). These vessels are of host origin, pierce the islet and branch into capillaries within the centre of the graft(*138*). Furthermore it appears that a physiological "core-to-mantle" perfusion is reinstated for optimal intercellular beta-to-alpha/delta sensing and signaling for optimal insulin and glucagon control(*139*).

Similar techniques of islet isolation and purification were not successful when applied to the more dense and fibrous pancreas of larger animals including the human gland. Mirkovitch *et al* were the first to reverse pancreatectomy-induced diabetes by intrasplenic autotransplantation of partially digested pancreatic tissue in dogs(*140*); intravenous glucose tolerance tests were indistinguishable from normal controls, even if less than half the pancreas was used for tissue digestion. Warnock and others subsequently showed that islet autografts prepared by enzymatic digestion and mechanical dispersion could reliably reverse the diabetic state in dogs(*141*). Griffin *et al* further showed that up to three recipients could be normalized by one donor graft when non-purified pancreatic tissue was infused intra-splenically(*142*). Unfortunately the human spleen is not distensible as in the dog, where the spleen serves as an important role in auto-transfusion in the face of life-threatening hemorrhage. Attempts to transplant impure or partially purified tissue intra-splenically in humans have met with considerable morbidity, including splenic rupture(*143*), wedge splenic infarction and portal vein thrombosis in a high proportion of recipients, although insulin independence has been achieved in the autograft setting(*144*).

Investigators resorted to intra-portal transplantation of impure pancreatic homogenates in dogs and ultimately in humans, leading to disastrous outcome including disseminated intravascular coagulation, portal vein thrombosis and the sequelae of portal hypertension, hepatic infarction and liver failure in some cases(*145-147*). Mehigan *et al* found that the addition of heparin and aprotinin to the tissue preparation at the time of transplantation could ameliorate the risk of disseminated intravascular coagulation(*148*).

Recent progress has occurred in the science of islet isolation, based on evolution of an enzymatic pancreatic dissociation process that provides more consistent high yields of viable human islets for transplantation. The techniques used currently evolved in a strong international collaborative effort with a select number of islet isolation laboratories. The early history and development of the current state of the art isolation methods are reviewed below. Recent methods have increased the efficiency of the process, and have had major impact in enhancing the consistency and quality of highly purified islet preparations for safe transplantation into patients. The evolution towards current techniques is outlined below.

Improvement in the isolation and purification of islets from the large animal pancreas became a major focus of intensive study in several laboratories, using the canine pancreas as the pre-clinical model. Intraductal injection of collagenase directly into the pancreatic duct was shown by Horaguchi and Merrell, and subsequently by Noel *et al* , to be the most effective way to dissociate the pancreas for high yield islet isolation, with up to 57% recovery of the total islet mass(*149, 150*). Trans-ductal collagenase delivery, whether by direct injection(*151*) or continuous perfusion(*152, 153*), was able to cleave the islet-acinar interface more readily than any method described previously, but still led to significant islet destruction through inadvertent islet enzyme penetration(*154*). However, the process did permit successful isolation of islets from the pig(*155*), monkey(*156*) and human pancreas(*151*).

The approach was further refined to allow precise control of the temperature and perfusion pressure(*157*). Lakey *et al.* subsequently demonstrated that retrograde intraductal Liberase-HI delivery using a recirculating controlled perfusion system provided superior human islet recovery and survival when compared to syringe loading(*158*). By providing control over perfusion pressures and Liberase temperature during loading of the enzyme into the pancreas, the recirculating controlled perfusion system more effectively delivers the Liberase to the interface of the islet-acinar interface resulting in a greater separation of islets from the surrounding exocrine tissue(*158*).

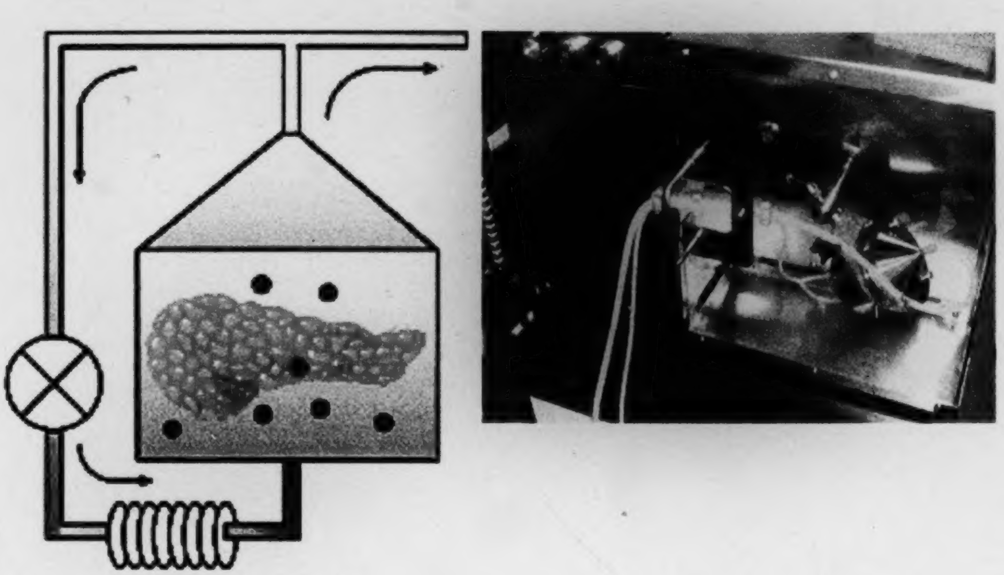


Figure 1.8: Ricordi continuous digestion chamber, and automated shaker

A major advance came with the introduction of a semi-automated dissociation chamber and process originally developed by Ricordi *et al* in 1988, modifications of which have now become the universal standard for successful high yield large animal and human islet isolation(*159*). The collagenase-distended pancreas is placed inside a stainless steel chamber containing glass marbles and a 500µm mesh screen and mechanically dissociated by gentle agitation, with tissue samples evaluated sequentially to determine the end-point before liberated islets become fragmented by over-digestion. This novel approach minimized trauma to the islets in a continuous digestion process with the collection of free islets as they are liberated from the digestion chamber. A comparison of manual and automated methods of islet isolation clearly demonstrated superiority of the automated method(*153, 160-162*). Since the introduction of this technique, many laboratories around the world have utilized this system for the isolation of islets from canine, pig and human islets.

The Large-scale purification of human islets of suitable quality for safe transplantation into the human portal vein was enhanced considerably by the introduction of an automated refrigerated centrifuge system (COBE 2991) by Lake *et al*, which permitted rapid large volume Ficoll gradient processing in a closed system 600ml bag(*163*). When Ficoll is made up in Euro-Collins solution (Euro-Ficoll), hypertonic exposure of the exocrine component reduces osmotic swelling and enhances differential islet-exocrine density improving purification, but results in significant b-cell stress with degranulation and loss of insulin content(*164, 165*).

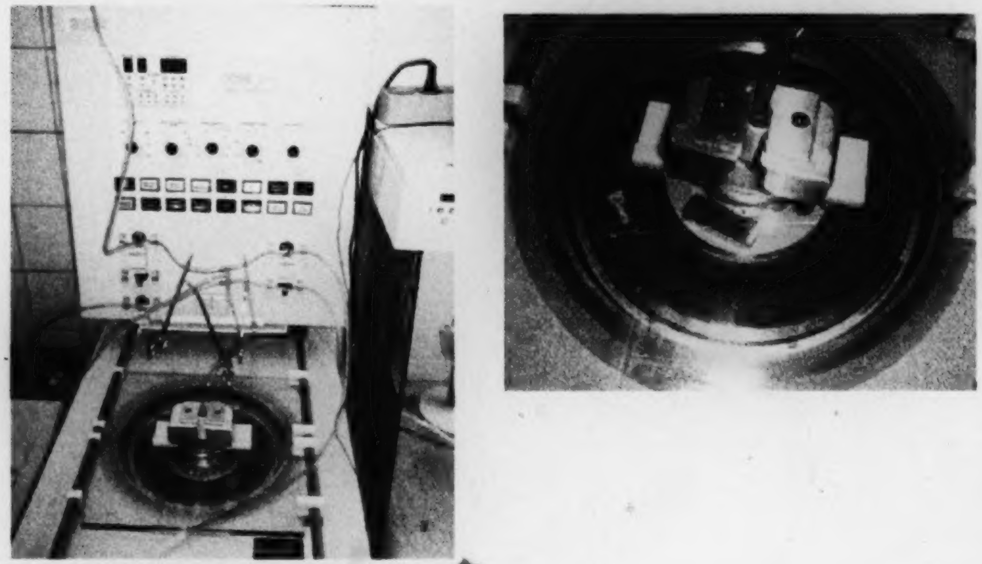


Figure 1.9 (a): COBE 2991 cell apheresis system

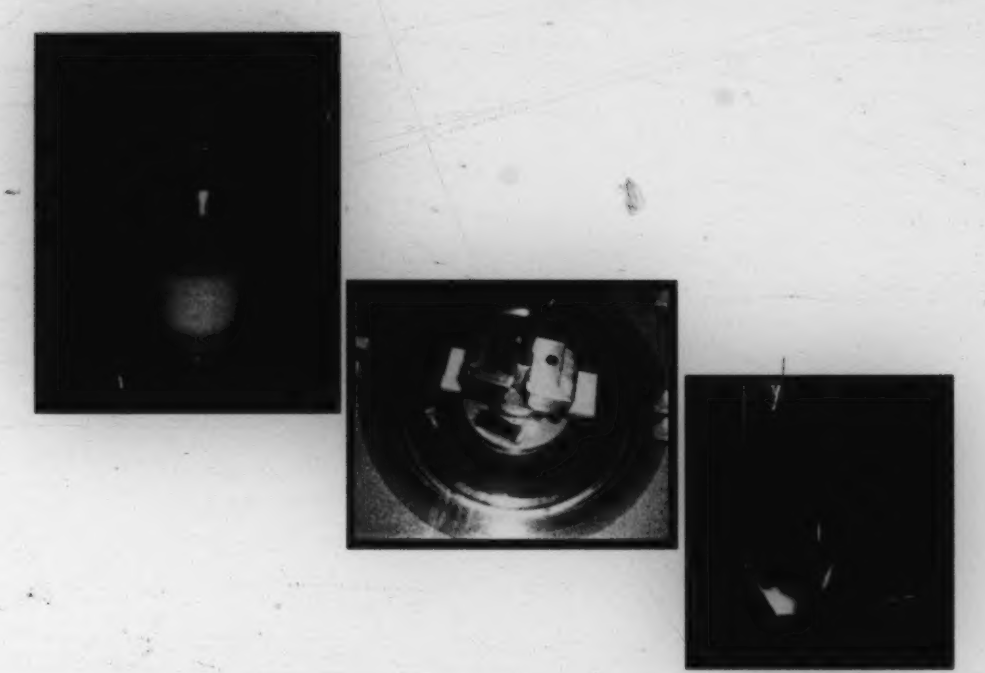


Figure 1.9 (b): Final packed cell volume is less than 4 cc's after purification.

A major limitation to successful pancreatic digestion up till recently has been the source, quality and variability in collagenase activity and contaminants in various enzyme blends. A new class of highly purified enzyme blend (Liberase^{TM}, Roche Pharmaceuticals, Indianapolis, USA) containing collagenase I, II, thermolysine, clostripain and clostridial neutral protease and with low endotoxin activity has provided consistently enhanced islet yield, viability and function from the human pancreas, and has been an important advance to the field(*166-169*). Refinements in manufacture have largely eliminated the lot-to lot variability in crude enzyme effectiveness for islet isolation(*168, 169*). Liberase has proven to be superior to crude collagenase preparations by consistently yielding large numbers of islets without compromising the functional viability(*169*).

Figure 1.10: Low-endotoxin Liberase™ collagenase – significant improvement in consistency and yield *(blend of Type I, type II collagenase with thermolysine. The white powder is the low-endotoxin Liberase, and the brown powder is the crude original collagenase preparation.)*

Despite the key advances in collagenase quality, intra-ductal enzyme delivery, automated dissociation and purification outlined above, inconsistency remains in the overall success of the islet isolation procedure, which may reflect variability in donor-related factors (donor inotropic need, duration of cardiac arrest, hyperglycemia, age and obesity in the donor, in addition to the skills of the local procurement team)(*170*).

1.6 CURRENT AND FUTURE DIRECTIONS IN EXPERIMENTAL ISLET TRANSPLANTATION

The ultimate goal of islet transplantation is to completely correct the diabetic state from an unlimited donor source, without the need for chronic immunosuppressive drug therapy. If this could be achieved with minimal risk to the recipient, its application in the earliest phase of Type-1 diabetes is likely to have the biggest impact on prevention of secondary diabetic complications. An islet transplant is privileged compared with other solid organ transplants as the graft may be stored in tissue culture(*171, 172*) or cryopreserved for banking(*173*), and has the unique potential for manipulation *in vitro* to alter graft immunogenicity.

A number of strategies have been developed to circumvent islet graft immune destruction, including graft immunomodulation, immunoisolation or induction of specific unresponsiveness. Inactivation of MHC class II passenger dendritic cells through low-temperature high-oxygen culture(*174, 175*), cryopreservation(*176*), gamma irradiation(*177*), ultraviolet light exposure(*178, 179*) or through *in vitro* addition of anti-class II antibody(*180*) are all strategies which have proven to be effective in rodents to prevent rejection, but have thus far been ineffective in larger animal models unless supportive chronic immunosuppression is given(*181, 182*). Attempts to transplant human islets cultured for 7 days at 24°C into diabetic human recipients with temporary immunosuppression resulted in early C-peptide production, but uniform rejection within two weeks of transplantation(*183*).

1.6.1 ISLET IMMUNOISOLATION

The immunoisolation of islets within micro-capsules initially promised to prevent allograft and xenograft rejection by elimination of cell-to-cell contact(*184*), but a more recent understanding of cytokine-mediated graft destruction would suggest that complete protection by porous barriers may be a more difficult hurdle to overcome without additional immunosuppression if insulin, metabolites and toxins are also to diffuse freely. There has been varied success achieved in rodent models, with some reports demonstrating long-term success(*185-188*), while others have been frustrated by intense fibrotic reaction which, in some cases has been induced by the alginate carrier, while in other cases has clearly been induced by shed graft antigens(*189, 190*). Very few studies have been able to demonstrate unequivocal protection against autoimmune diabetic recurrence in the NOD mouse by microencapsulation, unless combined with immunosuppressant drug or monoclonal strategies(*191-196*). One clear benefit of islet microencapsulation is in providing an efficient microenvironment for optimal islet metabolic function, and Ao *et al* have demonstrated a four to eight-fold improvement in islet function for encapsulated islets over their non-encapsulated counterparts both *in vitro* and when transplanted intra-peritoneally in nude mice(*197, 198*). Encouraging reports of sustained insulin independence beyond 9 months after intraperitoneal implantation of microencapsulated allogeneic islets in spontaneously diabetic dogs(*199*) and recently in a diabetic patient indicate that encapsulation technology has huge potential for clinical islet transplantation(*200*). The huge potential of microencapsulated xenograft islets has been enhanced by a recent report in which monkeys with spontaneous autoimmune diabetes given adult porcine alginate-encapsulated islets were normoglycemic for up to 803 days without any adjunctive immunosuppression(*201*).

Weir *et al* have recently reported prevention of diabetes recurrence beyond 300 days in autoimmune NOD mice using a barium-alginate capsule. Elimination of the selectively permeable poly-L-lysine component may have been key to avoiding inflammatory reactivity(*202*). These capsule will shortly be evaluated in primate allograft and xenograft models.

1.6.2 TOLERANCE INDUCTION IN ISLET TRANSPLANTATION

Tolerance strategies, the long sought-after *'Holy Grail'* of transplantation, will likely be developed first in islet transplantation. The consequence of failure of efficacy of a tolerance treatment would simply result in a patient's return to insulin, in contrast to the potential loss of a life-sustaining graft such as a heart or liver transplant that could precipitate patient demise. It is apparent that any approach that is successful in achieving tolerance to an islet allograft must also be effective in controlling recurrence of autoimmune diabetes.

Induction of tolerance through mechanisms of active specific unresponsiveness has been achieved in rodent models of islet transplantation, but until recently have not been successful in large animal or human models. Initial studies explored "immuno-privileged" sites for islet implantation including the testis(*203*), brain(*204*) and thymus(*205*), with marked prolongation or indefinite islet survival. It has become increasingly apparent that the immuno-protective benefits of these and other sites extend beyond a simple blood-tissue barrier property, and likely invoke anti-apoptotic pathways including but not restricted to Fas—Fas-ligand interaction(*206*). With respect to the testis, it appears that the sertoli 'nurse' cell is responsible for the local immunosuppressive properties of this site, leading to the potential for co-transplantation of sertoli and islet cells together for immunologic protection(*207, 208*). While promising in rodent models, it remains to be seen whether this approach will be effective alone in prevention of allograft and xenograft islet rejection in large animal or human studies.

The recent discovery that insulin is not only produced in the pancreas but also in small amounts in the thymus has important implications in the pathogenesis of Type-1 diabetes, in that higher levels of thymic pro-insulin expression may lead to negative selection of insulin-specific cytotoxic T lymphocytes with enhanced protection against the disease(*209*). This may explain in part why autoimmune diabetic NOD mice are protected against primary disease by islet implantation within the thymus(*210*). Donor specific bone marrow intra-thymic implantation also has been shown to induce donor-specific non-responsiveness by thymic T-cell negative selection in mice and rats after islet allograft transplantation, when augmented by single-dose anti-lymphocyte serum(*211*). One major limitation of intra-thymic immune manipulation is its clinical application, since most individuals with established diabetes have age-related thymic atrophy, and alternative sites for positive and negative T-cell selection in adult humans have been poorly characterized to date. Clinical attempts to inoculate the thymus with a portion of the islet transplant mass via CT guided percutaneous puncture have met with very limited success and no insulin independence thus far, despite transplantation of a substantial islet transplant mass (1,900,000 islet equivalents derived from seven donors) combined with induction and maintenance immunosuppression(*212*).

Interest in peripheral infusion of donor specific bone marrow cells followed the discovery that high level microchimerism of donor immune cells within the recipient could be induced by bone marrow transplantation. The degree of microchimerism appeared to be an important factor in attainment of stable non-responsiveness in a few long-term human liver and kidney transplant recipients who had been withdrawn from all immunosuppression for a variety of reasons(*213, 214*). Ricordi *et al* initially showed in a rodent islet allograft model that donor specific peripheral bone marrow transplantation without marrow ablation led to high level donor microchimerism with non-responsiveness to islet allografts(*215*). Current trials of donor bone marrow infusion combined with solid organ or islet transplantation are in progress to determine whether donor-specific tolerance can be achieved, and definitive results are eagerly awaited(*216, 217*). Preliminary data in clinical liver transplantation suggest that the combined high dose bone marrow in the presence of immunosuppression significantly decreases the incidence of rejection episodes and enhances liver allograft survival(*218*).

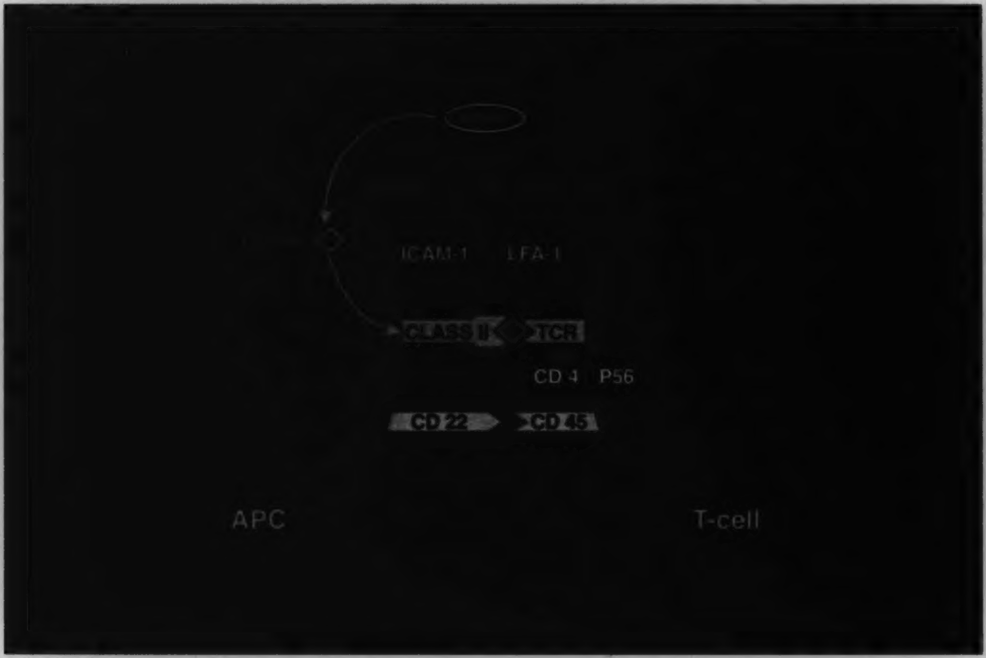


Figure 1.11: T-cell MHC-peptide engagement and costimulatory surface interactions with antigen presenting cells (APC's)

One of the most promising recent avenues in tolerance induction with potential clinical application has been through monoclonal antibody blockade of co-stimulatory second signal molecules in the early inductive phase after transplantation, to prevent 'Signal 2' activation while leaving 'Signal 1' T-cell receptor antigen engagement unaltered. The discovery by Linsley *et al* in 1991 that the fusion protein CTLA4-Ig, with its high affinity binding to B7 molecules, could uncouple second signal interaction and prevent activation of T and B cells through inhibition of CD28 - B7.1 / B7.2 on T and B cells respectively, opened up new frontiers for tolerance induction(*219, 220*).

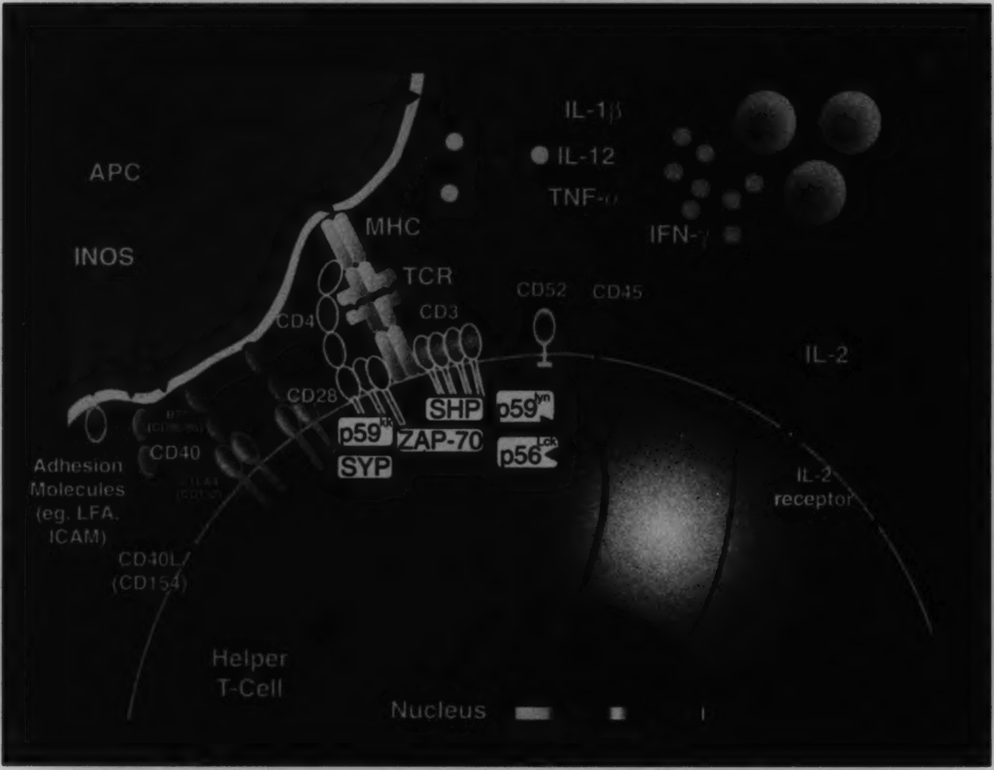

IL-1β
IL-12
TNF-α
IFN-γ
APC
INOS
MHC
TCR
CD3
CD4
CD52
CD45
IL-2
CD28
SHP
p59lyn
p59kk
ZAP-70
SYP
p56Lck
CD40
Adhesion Molecules (eg. LFA. ICAM)
CD40L/ (CD154)
IL-2 receptor
Helper T-Cell
Nucleus

Figure 1.12: Costimulatory and proximal T-cell activation pathway events

Numerous studies followed, demonstrating that systemic CTLA4-Ig could substantially prolong allografts and xenografts in rodent models of cardiac(*221*), renal, small bowel and lung transplantation, but in most cases efficacy was most marked when additional strategies of donor specific transfusion, bone marrow transplantation or low dose immunosuppression were combined with CTLA4-Ig(*222-224*). Enhanced survival of islet allografts(*225*) and xenografts(*192, 226*) has been demonstrated after systemic CTLA4-Ig administration. Strategies for local delivery of CTLA4-Ig, either by co-transplantation of CTLA4-Ig secreting myoblasts(*227*) or by gene-gun biolistic delivery of naked DNA gene for CTLA4-Ig(*228*) have been shown to considerably enhance islet allograft survival in diabetic mice. Recent studies of human CTLA4-Ig in non-human primate islet allografts led to prolonged graft survival in two of five monkeys, with suppression of both humoral and cellular immune responses(*229*).

The interaction of CD40 with its T-cell ligand CD40L has also been demonstrated to play a key role in T cell activation, in part by direct up-regulation of B7 molecules and also by enhancing the avidity of T-B cell interaction(*230, 231*), and this interaction is inhibited by the monoclonal antibody anti-CD40L (anti-CD154). Larsen *et al* found that in murine skin allograft transplant models, treatment with either CTLA4-Ig or anti-CD40L alone was insufficient to prevent long-term engraftment, but the co-administration of both co-stimulatory inhibitors led to dramatic synergistic interaction, and long-term survival of fully allogeneic grafts(*232*). Furthermore, Zeng *et al* have shown that these agents are effective in preventing antigen sensitization in murine islet transplantation, which may have important implications for clinical islet transplantation where multiple donors may have to be used to achieve insulin independence(*233*). In a primate renal allograft rejection model, Kirk *et al* found that administration anti-CD40L or CTLA4-Ig significantly prolonged graft survival, but co-administration of both agents led to indefinite survival and non-responsiveness to subsequent skin allografts from the original donor primate strain(*234*). Kenyon *et al* carried out intra-portal islet allografts in pancreatectomized diabetic monkeys treated with anti-CD40L, with five of six animals demonstrating insulin independence beyond one year(*235*).

While promising in large animal primate models, further testing of one potent anti-CD40 ligand blocking antibody (Hu5C8) has been halted due to unexpected thromboembolic complications in early clinical trials in rheumatoid arthritis that culminated in a patient death(*234, 236, 237*). This complication has not been identified in patients treated with a different anti-CD40L mAb (IDEC); this differential toxicity with an apparently similar antibody may reflect differential blocking vs partial stimulating effects of the antibody to its ligand with the Hu5C8 and IDEC antibodies. Since CD40L is expressed on platelets, it is believed that the Hu5C8 mAb may have increased platelet binding and release reactions leading to an associated increase in thromboembolic events. Safety testing of these novel approaches has to be of paramount importance if applied to islet transplantation, as the risk-benefit ratio must reflect the fact that the underlying diabetic condition is not immediately life-threatening. If techniques to induce tolerance to allo-antigens fail to completely protect against autoimmune activation, a reasonable compromise may be to use a costimulatory blocking or bone-marrow conditioning adjuvant strategy in concert with very low-dose immunosuppression to diminish the risk of malignancy and infection to almost zero. Sirolimus monotherapy at low dose would be one obvious choice in this setting, since priming of activation induced cell death remains unimpaired in activated T lymphocytes, and is therefore 'tolerance-compatible'(*238, 239*).Glucocorticoid treatment may also interfere with active tolerance pathways(*240, 241*). Controversy persists in terms of how essential it will be to eliminate calcineurin inhibitor therapy in tolerance regimens, as a small number of patients have achieved tolerance to kidney allografts following donor bone marrow transplantation from living donors under the temporary cover of cyclosporine therapy(*242*).

It remains to be seen whether strategies that provide robust tolerance to alloantigens will also effectively control recurrence of autoimmunity in patients with diabetes. Experimentally, techniques to induce either central or peripheral tolerance have shown benefit, but the most promising approaches have used a combined approach to achieve mixed chimerism. The combination of total body irradiation with bone marrow transplantation and two doses of anti-CD40L antibody was able to induce donor-specific allotolerance without recurrence of autoimmunity with prolonged islet graft survival in overtly diabetic NOD mice(*243*). Graft function was maintained beyond 100 days with robust tolerance to donor-strain skin grafts in this model(*243*). Peripheral tolerance induced by a diphtheria-conjugated T-cell immunotoxin combined with an inductive course of deoxyspergualin was able to render streptozotocin-diabetic and spontaneously diabetic primates operationally tolerant to islet allografts and concordant closely-related xenografts; insulin independence was maintained beyond one year(*244-247*).

Based on the emerging data outlined above, clinical trials of tolerance induction in islet transplantation are on the verge of being initiated in selected centres, and the results of these pilot trials are eagerly awaited. However, cautious optimism must remain, as the safety of these monoclonal therapies has yet to be defined in terms of added risk of infection, post-transplant lymphoproliferative disorder or other malignancies, and the efficacy of this approach in prevention of autoimmune diabetic recurrence in human studies requires further elucidation.

1.6.3 THE LIMITED ORGAN SUPPLY AND ALTERNATIVE ISLET SOURCES

Clinical islet allograft transplantation represents one of several potential therapeutic options in the attempted cure of type 1 diabetes mellitus, aimed at prevention and stabilization of secondary diabetic complications, and freedom from insulin and dietary restriction. In its current form, life-long immunosuppression is required to prevent rejection, and the risk attendant to chronic immunosuppression must be balanced in every case against the potential risk associated with on-going diabetic care.

There are an estimated sixteen million diabetics in North America, one million of whom have Type 1 diabetes, representing approximately 0.31% prevalence in the general population(*4, 7*). The current rate of cadaveric organ donation in North America (approximately 18 donors per million population per year) will provide only 5,700 donor pancreata per year(*248, 249*). If it were possible to isolate sufficient islets from one third of these pancreases for single donor: recipient islet transplantation, and if one third of the Type 1 diabetic population were suitable for an islet transplant, then clinical islet transplantation could supply less than 0.57% of the needs of the total Type 1 diabetic population each year. With a further incidence of 30,000 new cases of Type 1 diabetes per year in North America(*4*), clearly there is an enormous short-fall in the availability of human allograft donors which will severely limit the broad applicability of this therapy in the cure of the Type 1 diabetic population.

It is imperative that trials of clinical islet allotransplantation continue however, as the principle remains to be proven in the human setting that a safe form of islet transplantation can routinely achieve sustained long-term independence from insulin, with stabilization of secondary diabetic complications. Once this is certain, incremental steps will further explore the utility of xenogeneic or clonally engineered islet replacement for clinical use.

It has long been recognized that if islet transplantation is to be widely applied in the treatment of Type-1 diabetes then alternative islet sources must be found. Future prospects of human cloning technology loom close with the recent successful cloning of sheep and cows(*250*), and might one day provide perfectly matched 'isogeneic' human islets for transplantation, but there are enormous and insurmountable ethical hurdles to face(*251-253*). The ultimate challenge in islet transplantation will be to develop alternate tissue sources that no longer rely on this scarce resource, and this will be essential if sufficient insulin-producing, glucose-responsive cells are to treat the 130 million patients with diabetes worldwide. Intensive research in the islet stem cell has already made huge strides, with the recent demonstration of new islet budding from ductal elements(*254*) and with stimulation of pancreatic stem cell proliferation using neogenesis-peptides such as INGAP(*255*).

Considerable progress has been made recently in genetic engineering of cell lines for insulin production for application in diabetes. One of the major challenges however has been in the induction of glucose-sensing mechanisms in these cell lines for physiological and appropriate control of insulin secretion. An excellent review by Newgaard *et al* outlines current progress in attenuated insulinoma cell lines, plasmid transfer of the glucose transporter GLUT-2, viral transduction of the insulin promoter/enhancer and insulin gene expression within hepatoma cell lines(*256*). Control of cellular rejection, prevention of transmission of malignancy and glycemic regulation without risk of hypoglycemia are issues that prevent immediate human application, but one day may offer a promising alternative approach in diabetic control.

Genetic engineering of hepatocytes to secrete a single-chain insulin analogue(*257*), genetic engineering of intestinal mucosal K-cells to secrete insulin in physiological response to hyperglycemia(*258*), or the reversal of diabetes in mice with transplantation of embryonic stem cells(*259*), all suggest that alternate sources are not far away.

The development of transgenic pigs, protected against human complement attack with decay accelerating factor or anti-CD59(*260-262*), have overcome hyperacute discordant rejection, but the hurdles of accelerated acute vascular and chronic rejection persist, with a need to provide more intensive immunosuppression than is required currently in human allotransplantation. The relevance of hyperacute rejection phenomena to islet xenotransplantation is uncertain, as islet cells are unique in expressing very little of the gal alpha(1,3)gal target epitope(*263*), and islets become revascularized entirely from recipient endothelial cells(*139*). Transplantation of fetal porcine islet clusters beneath the renal capsule of diabetic patients undergoing renal transplantation with standard induction immunosuppression and deoxyspergualin did not lead to insulin independence, but pig C-peptide was detectable in urine for up to 400 days post-transplant(*264*). Perhaps the combined approach of encapsulated transgenic islets will overcome xenogeneic barriers, but this remains to be proven. Successful isolation of the fragile adult pig islet has proven to be a major challenge for most islet laboratories(*155*), but the high yield isolation of neonatal pig islet clusters by Korbutt *et al* may be one answer to the donor shortage(*265*). However, neonatal porcine islets may be more susceptible to hyperacute xenorejection due to increased expression of the gal epitope on ductal pancreatic elements(*266*).

Enthusiasm for xenogeneic tissue sources for islet transplantation has waned recently as a result of concerns regarding zoonotic viral transmission of pig endogenous retroviruses (PERV). The fear of a PERV pandemic of similar magnitude to AIDS has likely been overestimated, but concerns have been fueled by recent reports demonstrating that PERV is transcriptionally active and infectious across species in vivo following transplantation of pig tissues, and that PERV may infect human cells *in vitro*(*267-269*). Transgenic pigs expressing human complement-regulatory proteins have been developed to overcome immediate destructive pathways, but an unacceptable degree of potent immunosuppression is still required (cyclophosphamide) to overcome accelerated acute and chronic rejection, limiting clinical applicability for the present, further dampening enthusiasm for this approach(*270*).

Prospects for the broader application of islet transplantation in type 2 diabetes must await developments in alternate tissue sources(*271*). Perhaps ten times more cells may need to be transplanted in type 2 diabetes to overcome the effects of peripheral insulin resistance.

1.6.4 LIVING DONOR ISLET TRANSPLANTATION – FUTURE POTENTIAL

A series of over 50 living donor segmental pancreas transplants have been completed at the University of Minnesota(*272-274*). Initial developmental experience suggested a modest increased donor risk of procedural complications, impaired glucose tolerance or more seriously, new diabetes induction in healthy donors followed long-term(*275*). More recently, more careful selection to avoid obese donors, those with pre-resectional impairment of glucose tolerance or those at increased risk of diabetes due to positive serological autoimmune antibody markers (ICA, GAD or mIAA) has largely eliminated this risk. Furthermore, recent developments in surgical technique including the potential for laparoscopic or hand-assisted retrieval, may enhance the palatability from a donor's perspective, provided technical complications such as pancreatic fistula are avoided. The natural extension of this technique would be to carry out islet transplantation from living donors, since the potential risk to the recipient should be considerably less than a segmental pancreas transplant. The challenge will be to deliver an adequate islet engraftment mass to secure insulin independence with the technique, since recipients of the Edmonton Protocol have typically required two or more entire donor pancreata to achieve a satisfactory metabolic result. One potential may be to consider infusion of unpurified or partially purified islet preparations, since this was the traditional technique used previously in successful human islet-autografted patients after total pancreatectomy for chronic pancreatitis. Early experimental studies in large animals suggested that up to three recipients might be successfully cured by unpurified islet grafts prepared from a single donor(*142*). While living donor islet transplantation offers a unique potential as an alternative source of human islets, the approach will likely remain controversial so long as a healthy donor is placed at potential risk from procedural complications.

1.7 CLINICAL TRIALS OF ISLET TRANSPLANTATION – PRELUDE TO THE EDMONTON PROTOCOL

As of January 2000, a total of 447 human islet allografts, and 3,185 fetal or neonatal islet allografts and xenografts have been carried out in 79 institutions over the past 20 years, as reported to the Islet Transplant Registry(*276-278*). An in-depth analysis of these results is poignant, since the excellent success of islet transplantation in small and large animals in the laboratory, and of human islet autotransplantation after pancreatectomy, stands in contrast to the striking lack of success of islet allotransplantation in the treatment of the Type-1 diabetic. Extrapolation of the Islet Transplant Registry data is predicted to have important implications for the future direction of clinical islet transplantation.

The first series of clinical islet allograft transplants in Type-1 diabetic patients immunosuppressed with azathioprine and corticosteroids were reported by Najarian *et al* in 1977, and followed shortly after reports of successful cure of diabetes by islet transplantation in rats(*279*). It was anticipated that human islet transplantation would supersede whole pancreas transplantation, which was associated with appalling morbidity and mortality rates in that era. While the initial attempts at islet transplantation appeared to be safe, these efforts were largely ineffective. Of 7 patients transplanted with dispersed pancreatic tissue into the peritoneal cavity or via the portal vein, no patient achieved insulin independence, although some were able to reduce insulin requirements for limited periods(*279*). The first C-peptide negative Type-1 diabetic to achieve sustained insulin independence by one year after islet transplantation occurred in 1978 in Zurich, Switzerland after single donor-to-recipient transplantation of non-purified islet tissue embolized to the spleen, simultaneous with kidney transplantation(*280*). Despite a number of anecdotal reports since 1979, only 35 Type-1 diabetic patients have attained insulin independence after islet allograft transplantation, according to data registered with the International Islet Transplant Registry as of December 1997(*281*).

Activity in clinical islet transplantation may be sub-divided into five categories: 1) islet autografts in patients undergoing total pancreatectomy, 2) islet allografts after total pancreatectomy, 3) islet allografts in Type-1 diabetic patients, 4) fetal islet allografts or xenografts in Type-1 diabetics, and 5) islet allografts in Type-2 diabetics. Success may be judged in terms of patient survival, graft survival (C-peptide production), attainment of insulin independence, effect upon glycemic control (glycosylated HbA_1C), overall quality of life, and impact upon secondary diabetic complications.

1.7.1 ISLET AUTOGRAFTS AFTER PANCREATECTOMY

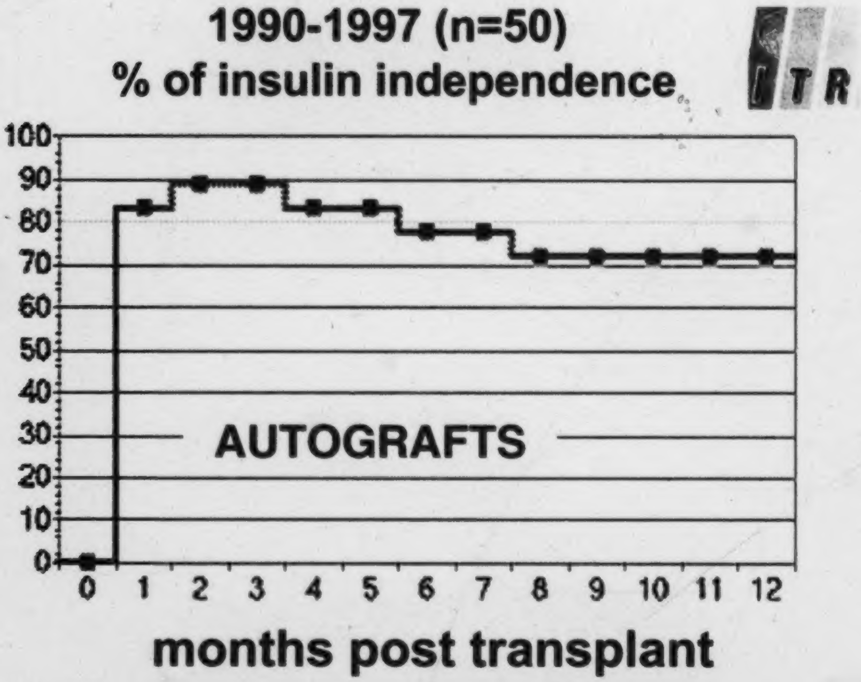


Figure 1.13: insulin independence rates after total pancreatectomy and islet autotransplantation *(Islet Transplant Registry data, provided with kind permission)*

The remarkable success of islet auto-transplantation has had major impact on overall progress and attitudes towards islet transplantation with the concept of insulin independence after islet transplantation in the clinical setting established beyond doubt. Indeed, the current literature suggests that after total pancreatectomy for chronic pancreatitis and intra-portal infusion of purified or unpurified pancreatic digest, approximately 50% of patients will be rendered independent of insulin. The first islet auto-transplant following pancreatectomy for chronic pancreatitis was carried out in

Minnesota in 1977(*282*), and over the subsequent 20 years a world experience has been accrued in 189 patients in 22 centres(*125, 281*).

Most patients underwent total or near-total pancreatectomy for intractable pain in chronic pancreatitis without pancreatic duct dilatation. Pyzdrowski *et al* reported a limited series of intra-portal islet autografts in whom all recipients became insulin independent after transplantation, and with documentation of functional intrahepatic islets on liver biopsy staining positive for insulin, glucagon and somatostatin, and with evidence of intrahepatic insulin secretion on hepatic vein catheterization(*283*).

Reviewing the experience of 69 islet autografts reported to the Islet Transplant Registry, 80% of patients became insulin independent for longer than one week, and 61% maintained insulin independence beyond one year(*281*). The longest follow-up of insulin independence in islet autografts is more than 13 years(*284, 285*). The best predictor of insulin independence in islet autografts is the number of islets transplanted, with a transplant mass exceeding 300,000 islets associated with an insulin independence rate of 74% at two years post-transplant(*286*). Farney *et al* further showed in a series of 29 islet autografts that 21% of patients lost graft function between 3 and 24 months after intraportal islet embolization where a median of 148,000 islets were transplanted, but if a median of 384,500 islets were given there were no late graft failures beyond 2 years, with a maximal follow-up of over 12 years(*287*).

Most centres have used non-purified pancreatic digest for islet autotransplantation because the fibrotic and atrophic nature of grafts scarred by chronic pancreatitis typically yield low tissue volume (usually 5mls or less). There is also concern that further purification of an already marginal islet transplant mass may render the exercise futile. While complications of portal vein thrombosis, disseminated intravascular coagulopathy and fatality have been described after islet autotransplantation previously, the risks have been minimized in recent years by systemic heparinization and better characterization of the dispersed grafts(*125, 148*). An accepted approach has been to Ficoll-purify pancreatic digests exceeding 15 ml in volume, to further lower the risk of portal vein thrombosis(*125*). The introduction of low-endotoxin collagenase (Liberase^{TM}) may also be critical in minimizing the acute risk of physiological perturbations associated with infusion of non-purified islet preparations.

While many different sites have been tried for islet autotransplantation, the optimal site appears to be through portal venous embolization. Attempts to embolized to the spleen led to significant life-threatening complications of splenic infarction, rupture and even gastric perforation(*144*).

1.7.2 ISLET ALLOGRAFTS AFTER PANCREATECTOMY

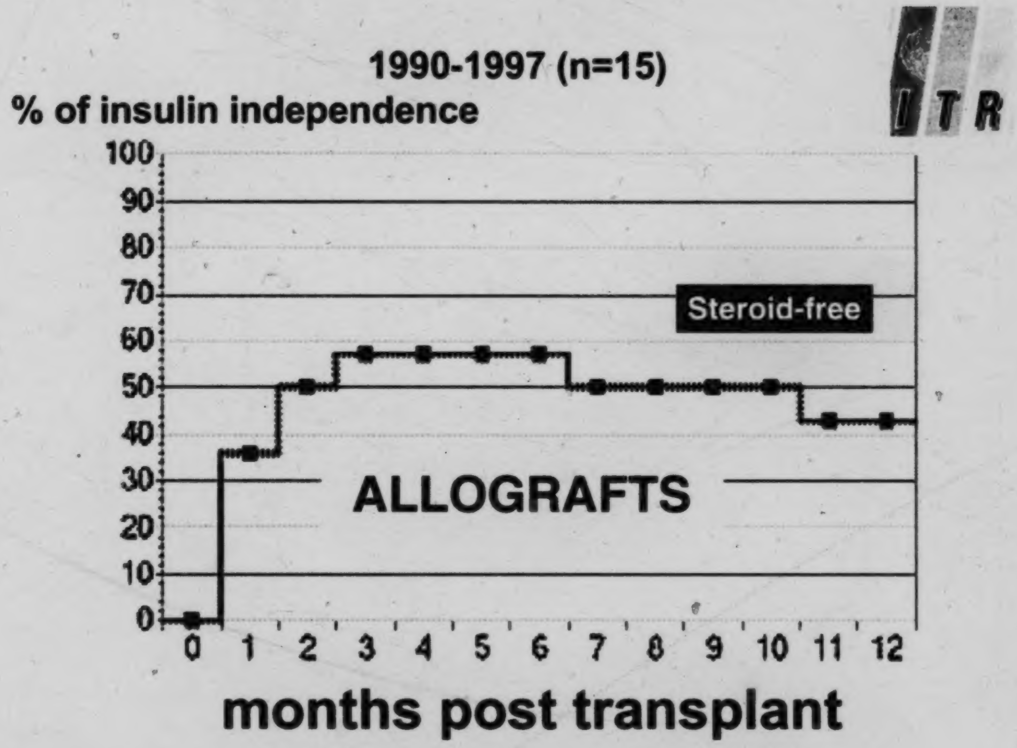


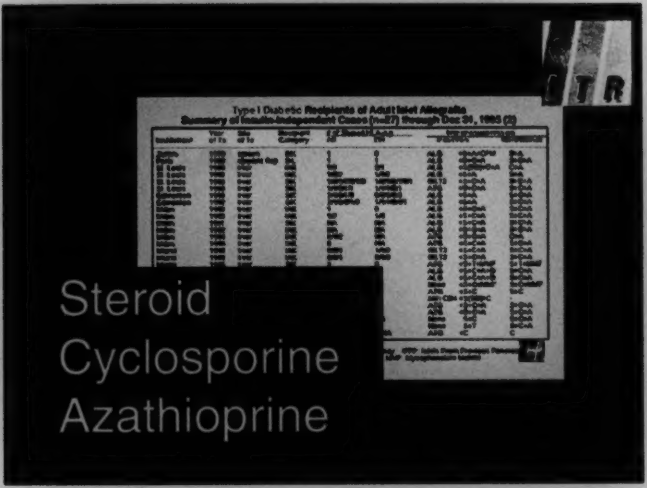
Figure 1.14: insulin independence rates after abdominal exenteration and cluster islet-liver-bowel allograft transplantation for malignancy – first experience with steroid-free immunosuppression *(Islet Transplant Registry data, provided with kind permission)*

A unique series of nine islet allografts were completed at the University of Pittsburgh in 1989 in patients undergoing abdominal exenteration with multi-visceral resection for malignancy followed by cluster transplantation of liver, kidney and bowel(*288*). Islets were isolated from a single multivisceral donor pancreas in the majority of cases, and infused intra-portally after liver reperfusion. Over 50% of recipients achieved and maintained insulin independence until their demise from recurrent malignancy. The series represented an unusual opportunity to complete islet allografts in the absence of an autoimmune diabetes background, which may have contributed to the preservation of the functional reserve of these grafts. Other major factors contributing to the success of the cluster-islet transplantation experience included: a) embolization of **partially purified** islet preparations and b) the use of **steroid-free immunosuppression (high dose tacrolimus monotherapy)** – which represented the first experience with less diabetogenic immunosuppression(*289*).

1.7.3 ISLET ALLOGRAFTS IN TYPE 1 DIABETES

A total of over 447 attempts to treat type 1diabetes with islet allografts were reported to the Islet Transplant Registry between 1974 and 2000, 394 of which occurred within the most recent decade(*276*). Mainstay immunosuppression was largely based on the combination of glucocorticoids, cyclosporine and azathioprine, with anti-lymphocyte serum induction(*290*). The majority of these grafts were combined islet-kidney transplants, since it was felt inappropriate to initiate new immunosuppression in islet-alone recipients who would not have otherwise required therapy to sustain another solid organ kidney or liver graft.

Under these protocols, fewer than 10% of patients were able to discontinue insulin therapy for longer than one year, although 28% had sustained C-peptide secretion at one year post transplant(*277*). These disappointing results contrasted with the success of islet autografts, and partial success of islet allografts in non-diabetic pancreatectomized recipients where glucocorticoid-free immunosuppression was combined with partially purified islet preparations(*289*).



Steroid
Cyclosporine
Azathioprine

Figure 1.15: Mainstay glucocorticoid-cyclosporine based diabetogenic immunosuppression for the majority of clinical islet transplants before the year 2000 *(Islet Transplant Registry data provided with kind permission; the background table illustrates that cyclosporine, glucocorticoid and azathioprine-based immunosuppression was used as mainstay therapy in most previous islet transplant attempts.)*

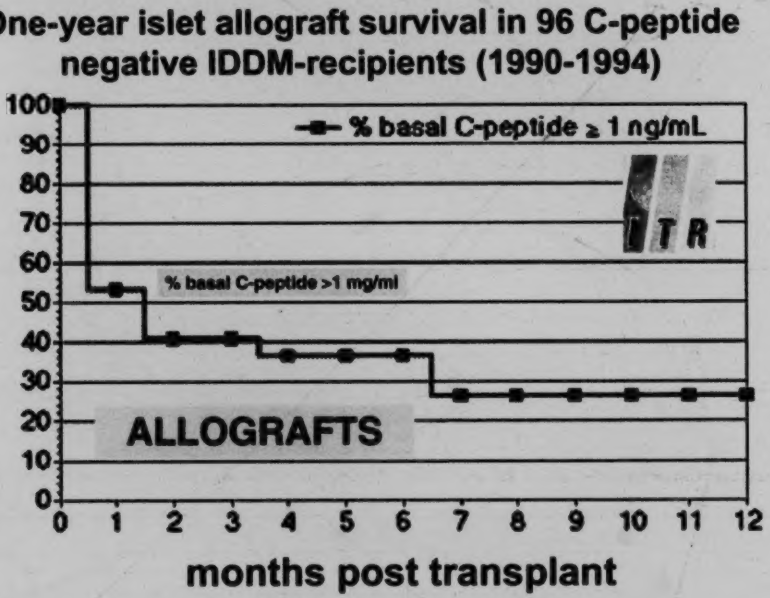


Figure 1.16: C-peptide production in type 1 diabetic recipients of islet allografts under cyclosporine, glucocorticoid and azathioprine-based immunosuppression *(Islet Transplant Registry data, with kind permission)*

A key question remains unanswered – are the previous poor results of islet allografts in Type 1 diabetic recipients a result of poor control of allo-immune pathways, or do they reflect recurrence of autoimmune diabetes? Insulin independence was only rarely achievable under glucocorticoid and cyclosporine-based immunosuppression. C-peptide secretion diminished to zero over time in most cases, suggesting islet graft loss from acute rejection or possible recurrence of autoimmune diabetes. Results of whole pancreas transplantation indicate that stable graft function is achievable over time, even with lower dose maintenance immunosuppression, suggesting that prevention of autoimmune destruction might be more readily achieved than prevention of allo-immune rejection. Autoimmune recurrence after whole pancreas transplantation only appears to be a challenge when no immunosuppression is given, as occurred in a living-donor hemi- pancreas transplant between identical twins, where autoimmune recurrence led to graft loss within two months *(313, 314)*.

Detailed analysis identified four "common characteristics" associated with improved success (cold ischemia < 8 hours, transplant mass > 6,000 IE/Kg, intraportal deliver and ALG/ATG induction but not OKT3)(*278, 291*); 29% of this sub-group were independent of insulin, and 46% had HbA_1C levels of less than 7%, which in the context of the DCCT trial, suggests that tight glycemic control afforded by islet transplantation might slow progression of secondary diabetic complications(*110*).

Recent results have improved in the past two years under cyclosporine, glucocorticoid and azathioprine immunosuppression, together with anti-IL2 receptor induction and anti-oxidants, with combined data from the Giessen and Geneva (GRAGIL consortium) groups reporting a 50% rate of C-peptide secretion and 20% insulin independence rate at one year(*292, 293*). Islets were cultured for a mean of two days, and mean islet implant mass was 9,000IE/kg, derived from single donors in half of cases. Two of ten patients achieved insulin independence after single-donor islet infusions, but it took 6-8 months to achieve independence, and both were recipients of shipped islets from a central islet isolation site(*292*).

The University of Milan recently reported experience with two immunosuppressant protocols in type 1 diabetic islet after kidney recipients (anti-lymphocyte serum (ALS) + cyclosporine + azathioprine + prednisone in the first Era (1989-1996) vs. anti-thymocyte globulin (ATG) + cyclosporine + mycophenolate + metformin together with anti-oxidants in the second Era (1998-2001)(*294*). Rejection rates were low in both eras (3/21 vs. 3/20 in Era one vs. two respectively). Rates of insulin independence were enhanced from 33% to 59% with the elimination of prednisone, and addition of mycophenolate and metformin. Over 50% of patients maintained insulin independence beyond one year with the newer protocol, possibly as a result of more effective immunosuppression coupled with anti-inflammatory, less diabetogenic and improved insulin action with the newer protocol.

1.7.4 FETAL ISLET ALLOGRAFTS OR XENOGRAFTS IN TYPE-1 DIABETES

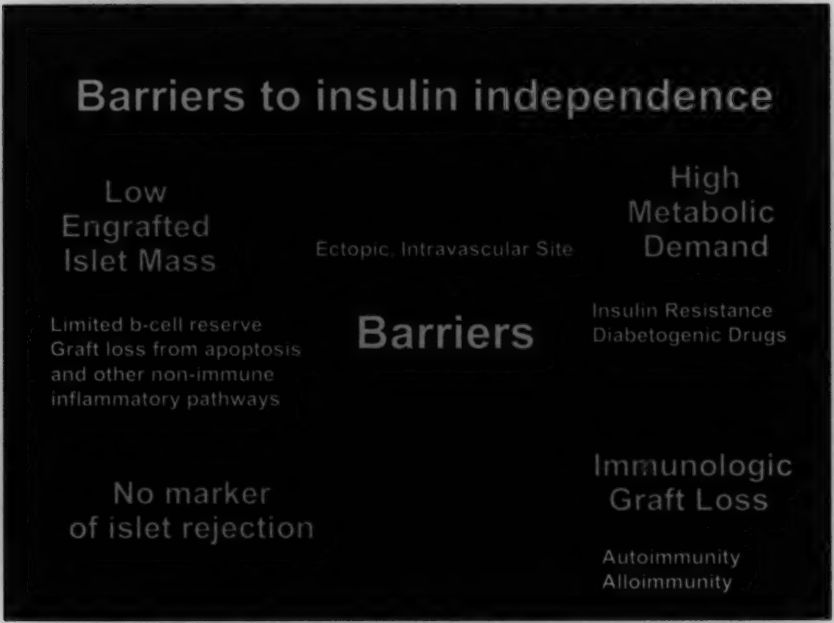
The total number of fetal and neonatal islet allografts and xenografts performed in human Type-1 diabetic recipients actually exceeds the number of adult islet allografts by a factor of ten times. A total of 3,185 cases have been published or registered since 1977, but the true cumulative total is now estimated to exceed 5,000(278, 281). Access to human fetal tissue is clearly more readily available in China and Eastern Europe, where over 96% of these transplants have been carried out. Turchin et al reported on their experience in 1,500 human fetal and neonatal porcine islet transplants carried out in Kiev, and found a reduction in hypoglycemic episodes(295). Insulin independence after human fetal or neonatal dispersed pancreas tissue transplantation was reported by Hu et al in 48 recipients from 54 hospitals in China, with delayed progression of microvascular secondary complications in patients with good graft function after 29 months of follow-up(296, 297). Insulin independence after human fetal islet transplantation has been reported in 9 further recipients in other centres(298-300). Unfortunately, despite this extensive experience these apparently successful outcomes must be interpreted with caution, as the majority of grafts have been poorly characterized in terms of transplant mass and pre-transplant C-peptide negativity. Tuch et al recovered human fetal islet grafts with persistent beta cells in three patients between 9 – 14 months after transplantation, but could not demonstrate immunoreactive C-peptide in peripheral blood and found histological changes of islet rejection(301). Groth et al detected porcine C-peptide in the urine from 200 to 400 days after transplantation in four of ten patients transplanted with fetal porcine islet clusters, but could not document C-peptide in serum(264). Some investigators used non-human xenogeneic islet tissue derived from bovine, porcine and rabbit sources, with implantation to a variety of sites including muscle, spleen, bone marrow and even direct intracerebral implantation(278, 302). Most of the transplants were performed without adjuvant immunosuppression. Based on current evidence, human fetal islet transplants are not protected from autoimmune attack(303). The issues of rejection(304), immaturity of the human fetal pancreas and ethical issues surrounding recovery of human fetal tissue remain significant challenges for this approach in the cure of diabetic patients.

1.7.5 ISLET ALLOGRAFTS IN TYPE 2 DIABETES

Up till recently it was believed that clinical islet transplantation would have no beneficial role in Type 2 diabetes, based on the understanding that the underlying metabolic defect in Type 2 disease is insulin resistance from abnormalities in insulin receptor number, function and post-receptor signaling. It is becoming increasingly clear though that beta-cell dysfunction may also co-exist in Type 2 disease(*305, 306*). Thomas *et al* reported encouraging data of islet transplantation in animal models of type 2 diabetes in the BB rat and NOD mouse(*307*). Ricordi *et al* further carried out combined islet-liver allografts in patients with Type 2 diabetes and liver cirrhosis, and in preliminary data demonstrated greater improvement in insulin requirement, HBA_1C and overall metabolic control than would have been expected by liver transplantation alone(*271*).

When vascularized pancreatic transplants have been carried out inadvertently in Type 2 diabetics, retrospective studies have shown surprisingly excellent pancreatic graft function with insulin independence(*308*). It is not clear whether whole pancreas transplantation is truly able to match the abnormal insulin demand caused by peripheral insulin resistance without b-cell exhaustion in Type 2 disease, or whether the patients inadvertently transplanted with high C-peptide levels are atypical, possibly with specific defects in the glucokinase gene. While it is expected that clinical islet and whole pancreas transplantation will remain focused in Type 1 diabetes, further studies are justified to determine whether a sub-group of Type 2 diabetics might benefit from endocrine replacement therapies.

1.8 SYNTHESIS OF WORLD EXPERIENCE IN CLINICAL ISLET TRANSPLANTATION – LESSONS LEARNED TO MOVE FORWARD WITH INNOVATIVE PROTOCOLS FOR INSULIN INDEPENDENCE



Barriers to insulin independence

Low Engrafted Islet Mass

Limited b-cell reserve
Graft loss from apoptosis
and other non-immune
inflammatory pathways

Ectopic, Intravascular Site

Barriers

High Metabolic Demand

Insulin Resistance
Diabetogenic Drugs

No marker of islet rejection

Immunologic Graft Loss

Autoimmunity
Alloimmunity

Figure 1.17: Barriers to the attainment of insulin independence after islet allograft transplantation in type 1 diabetes *(adapted from Hering and Ricordi: Graft 1999, with kind permission from the authors)*

The Islet Transplant Registry has provided an extremely valuable opportunity to critically study factors influencing rates of insulin independence after clinical islet transplantation. Islet transplantation as a theoretical and experimental concept has consistently provided insulin independence in small and large animal models of diabetes over the past 30 years. While patients undergoing total pancreatectomy with islet autograft or allograft infusions have yielded tantalizing and encouraging results, but achieving similar results in allograft recipients with type 1 diabetes has proven to be an almost insurmountable challenge until recently.

A recent review of the potential barriers to attainment of insulin independence after islet transplantation identified several factors leading to failure(*278*) These include:

1) An inadequate beta-cell reserve due to a limited islet engraftment mass, compounded by immediate cellular loss through apoptotic and other non-immune inflammatory pathways(*309-311*)
2) Immunologic graft loss from dual forces of attack – alloimmune and autoimmune pathways resulting from use of ineffective prophylactic immunosuppression(*312*)
3) An inability to diagnose early acute rejection events in islet grafts because of a lack of available tools, depriving the clinician of the opportunity to intervene with effective anti-rejection treatments
4) Excessive stress placed on islet grafts due to the high islet metabolic demand of pre-existing insulin resistance in patients with incipient renal failure, and compounded through the use of highly diabetogenic immunosuppressants – including calcineurin inhibitors and glucocorticoids given in combination.

The scope of the current thesis was to address most of the above barriers in a systematic fashion, with the ultimate goal of enhancing successful attainment of insulin independence after islet allograft transplantation in patients with type 1 diabetes. A critical review of available immunosuppressive agents facilitated prospective evaluation in large animal islet autograft and allograft models of diabetes. Parallel studies determined new methods for early diagnosis of islet rejection. A synthesis of these pre-clinical studies provided direction to move forward with a clinical trial of islet transplantation using a rationally designed but innovative protocol in an attempt to improve clinical results. The results of the clinical trial of islet-alone transplantation with the use of glucocorticoid-free immunosuppression will be presented in Chapter 7 and 8 of this thesis.

1.9 THESIS AIMS

This thesis addresses the major issues that have previously limited successful clinical implementation of islet transplantation for selected patients with autoimmune diabetes, with the ultimate goal of initiating a new clinical trial in islet transplantation. The over-riding hypothesis states that insulin independence will be routinely attained and maintained after clinical islet transplantation when a rationally designed protocol is developed from a logical series of interconnected pre-clinical experiments that build upon each other to reach this goal.

The specific aims of this thesis are therefore:

I. To identify specific deficiencies from a critical review of previous world experience in clinical islet transplantation, and to determine from the Islet Transplant Registry data what factors could be addressed to enhance rates of insulin independence (Chapter 1).

II. To review current state of the art immunosuppressant protocols to determine whether more potent but less diabetogenic regimen could be rationally designed to meet the specific needs of an islet graft (Chapter 2).

III. To determine diabetogenic toxicity of a selection of standard and new anti-rejection therapies when given alone or in combination (Chapter 3).

IV. To determine whether oral delivery of anti-rejection drugs leads to increased toxic exposure to islet grafts embolized through the portal vein (Chapter 4).

V. To evaluate three novel approaches to detect early rejection of islet grafts in a small animal model, at a stage before irreversible destruction has occurred (Chapter 5).

VI. To synthesize the results of all of the above pre-clinical studies, and to merge them with efficacy and safety data from large animal pre-clinical, clinical registry data and experience in management of solid organ transplant recipients, to design a new *"Edmonton Protocol"* to enhance the success of clinical islet transplantation in autoimmune diabetes (Chapter 6).

VII. To implement the new protocol in a series of islet-alone recipients and to determine outcome data of insulin independence and metabolic parameters of islet graft function in these recipients followed beyond one year (Chapter 7 and 8).

VIII. Based on data derived from all of the above studies, to draw firm conclusions, and from these determine a rationale approach for future directions in translational and clinical studies (Chapter 9).

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CHAPTER 2:

IMMUNOSUPPRESSIVE DRUGS FOR CLINICAL ISLET TRANSPLANTATION

NOTE: This chapter has been extensively updated and modified from a previous version of a book chapter by A M J Shapiro and N M Kneteman, originally submitted and accepted for publication in 1996, in *"Pancreatic Islet Transplantation for Human Diabetes: Challenges and Controversies"*,
Warnock GL Ed., John Wiley and Sons Ltd.

2.1 INTRODUCTION

Clinical organ transplantation entered a new era with the introduction of cyclosporine based immunosuppression. Quantum leaps in both patient and graft survival led to an explosive increase in numbers of transplants performed. Balanced immunosuppression, using multiple agents in combination to minimize toxicity, and by careful monitoring to prevent the consequences of over-immunosuppression (opportunistic infection and malignancy), has meant that kidney, liver and heart transplant recipients may now enjoy one-year actuarial survivals of 94%, 83% and 84% respectively(*1*).

Clinical islet transplantation, with its perennial promise of freedom from injected insulin and secondary diabetic complications, has thus far failed to deliver–of over 447 islet transplants reported to the Islet Transplant Registry, less than 10% achieved insulin independence at one year(*2-4*).

This limited success reflects a number of major drawbacks when conventional immunosuppression is applied to islet transplantation: Without a means to diagnose and thus reverse rejection of islet allografts, and based on the rejection rates of other solid organs under cyclosporine based immunosuppression, 40-70% of islet grafts will be destroyed by their first (and final) acute rejection episode within the first year(*5*).

Cyclosporine and steroids are synergistic in their diabetogenic potential, through decreased insulin secretion, increased insulin resistance and direct toxicity to the beta-cell; the incidence of new onset diabetes mellitus in organ transplant recipients receiving cyclosporine and steroids varies between 4% and 20%, and of these 40% will require insulin therapy(*6*). The negative metabolic effects of conventional immunosuppression are likely to be exaggerated in the islet transplant recipient, particularly if the engraftment mass is sub-optimal.

A limited islet engraftment mass may be insufficient to overcome the marked state of insulin resistance in the long-term uremic diabetic(*7, 8*).

Cyclosporine and steroids may interfere with islet neovascularization, further compounding the problem(*9*).

The future of islet transplantation holds promise–strategies directed at encapsulating islets behind semi-permeable membranes, *in vitro* immuno-alteration to deplete passenger leukocytes, genetic manipulation to induce local immunosuppression, transgenic islets protected from complement mediated attack, and methods to induce tolerance are all approaches which may one day allow widespread application of islet transplantation in IDDM, but are unproven at the present time. Non-specific recipient immunosuppression is thus likely to remain with us and be a critical component of therapy at least in the near future, and may be adjunctive to many of the above strategies. The search for newer, more specific and less toxic immunosuppressive agents continues, with the ultimate hope of inducing long-term tolerance of graft antigens while preserving response to third party antigens and microbes intact.

We will therefore review the array of established and novel immunosuppressive agents currently available, outlining their potency, synergy, toxicity, diabetogenicity and mechanisms of action. Based on this information, we hope to provide a rational approach towards optimally tailored immunosuppression to meet the specific needs of islet transplantation.

2.2 OVERVIEW

The therapeutic arsenal of immunosuppressive agents has expanded exponentially in the past 5–10 years, largely through developments in monoclonal technologies. The wide variety of immunosuppressive agents may be categorized in terms of their site of interaction with the various steps in the immune activation and amplification cascade; this may be at the level of antigen presentation, with inhibition of macrophage/monocyte function (deoxyspergualin), at the level of T helper cell response, with interference of signaling between the T-cell receptor and nuclear transcription of the IL-2 gene (cyclosporine, FK-506), cytokine action (rapamycin, leflunomide, glucocorticoids, anti-IL2R mAb), lymphocyte proliferation (azathioprine, mycophenolate mofetil, brequinar sodium) or lymphocyte depletion (OKT3, antilymphocyte globulin, cyclophosphamide). In addition, a number of monoclonal preparations directed against 'adhesion or accessory' molecules, have shown efficacy in the experimental setting, but await trials in clinical practice (non-mitogenic-CD3, anti-VCAM, anti-LFA-1, anti-ICAM-1, CTLA4Ig and mutant variants (binding B7 molecules and uncoupling second signal activation), etc.)

Most of the newer immunosuppressive agents show a clear trend towards more highly selective disarming of parts of the immune response. The original drugs, which crudely eliminated all lymphocytes, accounted for a considerable proportion of morbidity and mortality in transplant recipients. As our knowledge of immunology expands, so will the specificity of our therapies.

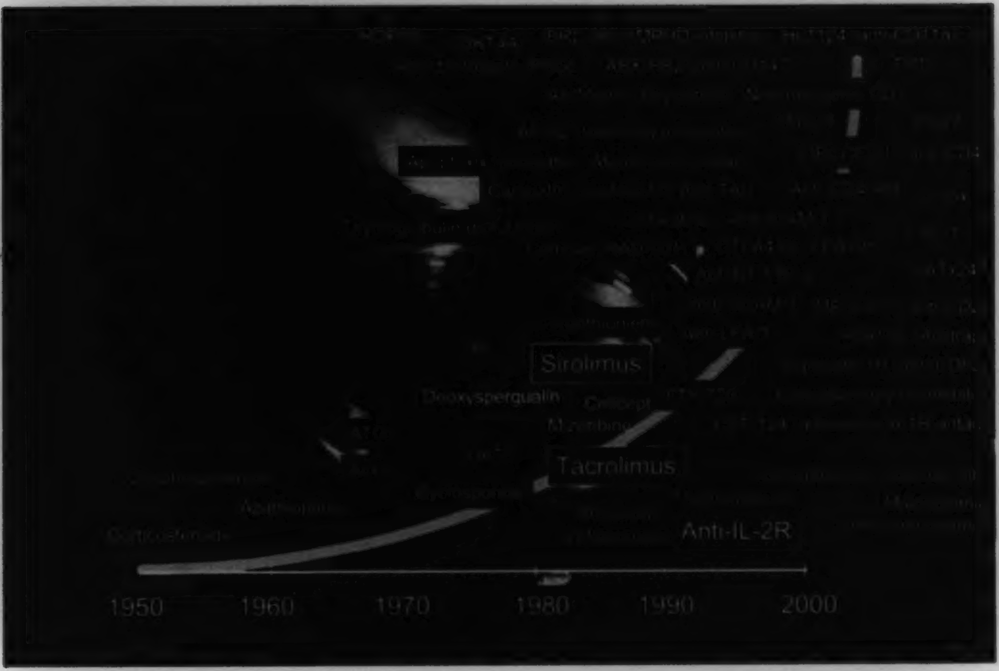
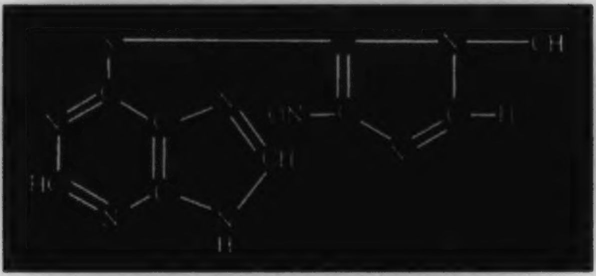


Figure 2.1: Explosive increases in availability of more specific, more potent anti-rejection therapies in current clinical practice or in early Phase I/II trials – suggesting that tolerance or near-tolerance may be an achievable goal.

2.3 THERAPEUTIC AGENTS

2.4 AZATHIOPRINE

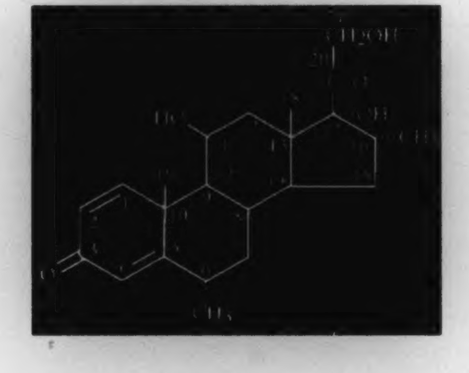


In 1959, Schwartz and Damashek discovered that an anti-cancer agent, 6-mercaptopurine, was able to suppress the immune response to human serum albumin in adult rabbits(*10*). In the following year Calne(*11*) and Zukoski(*12*) showed independently that this agent could prevent rejection of kidney transplants in dogs. Calne found that an imidazole derivative of 6-mercaptopurine, BW57-322, now known as azathioprine (Imuran), was equally effective but much less toxic, and its application to clinical renal transplantation changed the face of transplantation in the early 1960's(*13, 14*).

Azathioprine is a purine analogue that is converted *in vivo* to 6-mercaptopurine and 6-thio-inosine monophosphate. These block conversion of inosine monophosphate (IMP) to adenosine monophosphate (AMP) and guanosine monophosphate (GMP), and competitively inhibit lymphocyte proliferation, during the S phase of the cell cycle, largely through depletion of adenosine.

Given at a dose of 1-2mg/kg per day, azathioprine is frequently used as adjunctive immunosuppression to cyclosporine and steroids in clinical practice. Its efficacy at low dose in combination therapy was not confirmed by clinical trial until recently, when indirect evidence from control groups in studies of mycophenolate mofetil showed a reduced incidence of acute rejection at six months after kidney transplantation of 38% vs. 46% in patients receiving cyclosporine and steroids alone(*15*). Side effects relate to azathioprine's dose dependent effect on DNA synthesis, and include myelotoxicity and, more rarely, cholestasis; treatment dose is usually reduced by 50% if the white cell count is between 3.0 - 5.0 x 10^9/L, and discontinued in the face of systemic infection. Interestingly, azathioprine remains one of the few immunosuppressive agents that do not appear to have adverse impact on beta-cell function or on insulin sensitivity when used alone. It is a rather weak but widely used immunosuppressant, and its previous perceived benefit in islet transplantation has been in sparing the amount of steroid required.

2.5 GLUCOCORTICOIDS

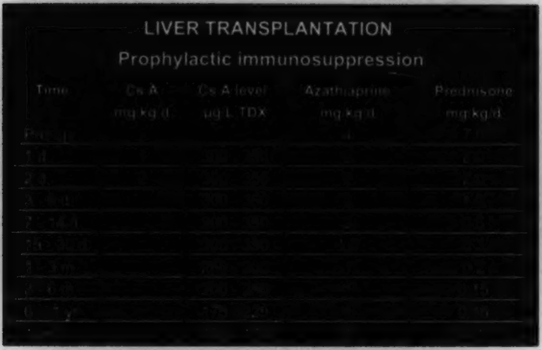


Corticosteroids are potent inhibitors of inflammatory responses and act at multiple stages in the rejection cascade. When given systemically steroids are toxic to lymphocytes, particularly immature cortical thymocytes, inducing programmed cell death by apoptosis. Corticosteroids also inhibit macrophage and other phagocytic cell activity by stabilizing lysosomal membranes, reduce chemotaxis and lower expression of class II MHC and IL-1, limiting response to activated T-helper cells. The lipophilic nature of steroids allows them to cross the cell membrane and bind to cytosolic receptors. These are transported subsequently to the nucleus where they bind to specific regulatory DNA sequences leading to up-regulation or down-regulation of transcription–predominantly of IL-1 and IL-6 gene transcription by macrophages, interfering with co-stimulatory signaling to T-helper cells, and thus indirectly blocking IL-2 release(*16*).

Corticosteroids are frequently used for synergistic immunosuppressive effect in combination with cyclosporine (or FK-506) and azathioprine as maintenance therapy. Since corticosteroids act at multiple sites, and suppress the entire immune system, they predispose to a multitude of side effects which are mostly dose dependent –increased susceptibility to systemic infection, steroid-induced diabetes (with major implications clearly for islet or pancreas transplantation), impaired wound healing, hypertension, cataracts, cushingoid habitus, osteoporosis, peptic ulceration, steroid-induced psychosis, acne, and growth retardation in children.

The diabetogenic effects of corticosteroids were first recognized by Ingle in 1941(*17*), and later by Starzl in kidney transplant recipients(*18*). The underlying mechanisms of steroid diabetes are multifold, the predominant defect being a reduction in insulin sensitivity(*6*); down-regulation of insulin receptors, reduced insulin receptor affinity, impairment of post-receptor signaling(*19*), reduced peripheral glucose uptake(*20, 21*), and altered glucose/free fatty acid cycle kinetics(*22*), have all been implicated. Using euglycemic-hyperglycemic clamp and radioactive glucose tracer techniques, Luzi found reduced insulin-stimulated peripheral glucose uptake in non-diabetic subjects treated with systemic steroids for chronic uveitis; combined kidney-pancreas transplantation in long-term type 1 diabetics normalized hepatic glucose production and improved insulin sensitivity–residual defects being secondary to chronic steroid therapy(*7*), and from systemic delivery of insulin(*23*). The negative effects of steroid therapy on insulin sensitivity and glucose effectiveness seen in human subjects may not be paralleled in pre-clinical models in the dog–Moore *et al.* found that oral treatment of over 1 mg/kg prednisone for a period of one month to normal dogs had no impact on insulin sensitivity or glucose effectiveness(*24*); similarly, we have found no evidence of negative impact after one month of steroid therapy in dogs bearing long-term intrasplenic islet autografts (see Chapter 3). The negative impact of steroids in humans is likely to be potentiated by cyclosporine, since both are cleared by cytochrome P-450 metabolism–Ost showed that prednisone clearance was significantly lower in renal transplant recipients treated with cyclosporine and steroids, compared with azathioprine and steroids(*25*).

A typical balanced immunosuppressive regimen (as used up till recently in our institution) used higher doses of drug initially, reflecting the fact that the risk of graft rejection is greatest in the first three months post transplant. As the graft becomes more readily accepted through a process called "accommodation", dose reduction can occur in parallel with the diminishing risk of acute rejection. Steroids are highly effective when given intravenously as bolus therapy to treat episodes of established rejection – typical doses in adult patients being 500 mg qd x 3 days, followed by a tapering oral dose of 125, 75, 40 and 25 mg/day.



LIVER TRANSPLANTATION

Prophylactic immunosuppression

Time	Cs A mg/kg/d	Cs A level µg/L TDX	Azathioprine mg/kg/d	Prednisone mg/kg/d

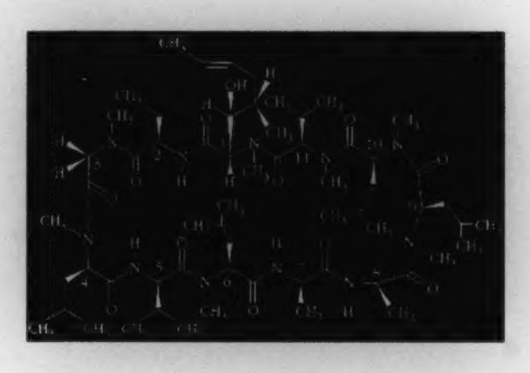
Table 2.1: A typical balanced immunosuppression regimen – demonstrating the concept of '*graft accommodation*' – as the risk of acute rejection diminishes over time.
(with kind permission from the University of Alberta Liver Transplant Program).

The multiplicity of steroid-related side effects has led many centres to taper and withdraw steroids early post transplantation. Initial experience in pediatric liver transplantation began with complete steroid and azathioprine withdrawal by three and 12 months respectively(*26*); the overall incidence of acute and chronic rejection was surprisingly low–24% at one year. Steroid taper and withdrawal is rapidly becoming an accepted approach in liver, kidney and heart transplantation(*27-34*), and has proven successful also in whole pancreas transplantation(*35*). The measurable benefits of this approach have been reduced rates of early infections, improved wound healing, reduced rates of osteoporosis and related fractures in adults, and improved growth recovery in pediatric recipients(*34*). Steroid withdrawal has been associated with reduced rates and better control of hypertension, reduced total cholesterol levels, reduced rate of post-transplant diabetes, improved control of diabetes, and reduced rate of obesity(*29, 30*). The aggregate experience with steroid withdrawal in liver transplantation suggests it is safe, associated with improvement in several post-transplant complications, and merits broader application. The risk of steroid withdrawal has been an increase in acute rejection incidence particularly after renal transplantation. Large recent meta-analysis steroid withdrawal trials in kidney transplantation indicate a 14% increased relative risk of acute rejection that may impart increased risk of late graft failure(*28*).

Complete glucocorticoid avoidance from the outset provides an attractive opportunity to minimize diabetogenicity in islet transplantation. Kneteman *et al* were the first to demonstrate detrimental effects of prednisone on canine islet autograft function in 1987(*36-38*). Early failure occurred in 2 of 6 canine islet autografts treated with glucocorticoids and azathioprine, in contrast to 0 of 10 failures in untreated controls(*36-38*). These finding that were confirmed subsequently by Kaufman, Zeng and others in allograft studies(*39-41*). The first clinical trial of steroid avoidance in islet transplantation was reported by Ricordi *et al* in patients undergoing cluster islet-liver replacement after abdominal exenteration – and may have been one of the main factors securing the high rate of insulin independence reported in that trial(*42, 43*).

Attempts to wean long-term transplant recipients from all immunosuppression have met with limited success in highly selected individuals; of a series of long-term (5-10 years post transplant) kidney and liver transplant recipients, 25% were able to discontinue all immunosuppression(*44, 45*). Complete withdrawal of all immunosuppression in islet transplantation will only succeed if both alloimmune and autoimmune destructive pathways have been neutralized by a successful tolerance protocol. A steroid free immunosuppressive regimen provides a unique opportunity to enhance function of islet allografts, but clearly this will only be possible if more potent and more specific newer immunosuppressants are given, thereby avoiding diabetogenic side effects. The availability of more potent drugs including tacrolimus, mycophenolate and now sirolimus may allow safer withdrawal of steroids(*46*). Glucocorticoid therapy may interfere with active tolerance pathways(*47, 48*). Controversy persists in terms of how essential it will be to eliminate calcineurin inhibitor therapy in tolerance regimens, as a small number of patients have achieved tolerance to kidney allografts following donor bone marrow transplantation from living donors under the temporary cover of cyclosporine therapy(*49*).

2.6 CYCLOSPORINE



Cyclosporine (CsA) is a cyclic endecapeptide derived from the soil fungus *Tolypocladium inflatum*. Borel discovered its potent immunosuppressive properties in 1976(*50*), and its introduction into clinical practice by Calne *et al* in 1979(*51*) revolutionized the results of solid organ transplantation. CsA blocks the clonal expansion of CD4+ve cells (predominantly T helper) by interference with the intracellular signaling pathway from the T cell receptor (TCR) to the cell nucleus, indirectly preventing transcription of the IL-2 gene. The loss of IL-2 expression in turn prevents further recruitment, proliferation and differentiation of effector T and B cells, and prevents augmented MHC expression and up-regulation of IL-2 receptor expression(*52, 53*), all pivotal steps in T helper response that would otherwise have initiated target cell injury.

Thus CsA is a potent inhibitor of initiation events, but is less effective in the treatment of an established rejection episode.

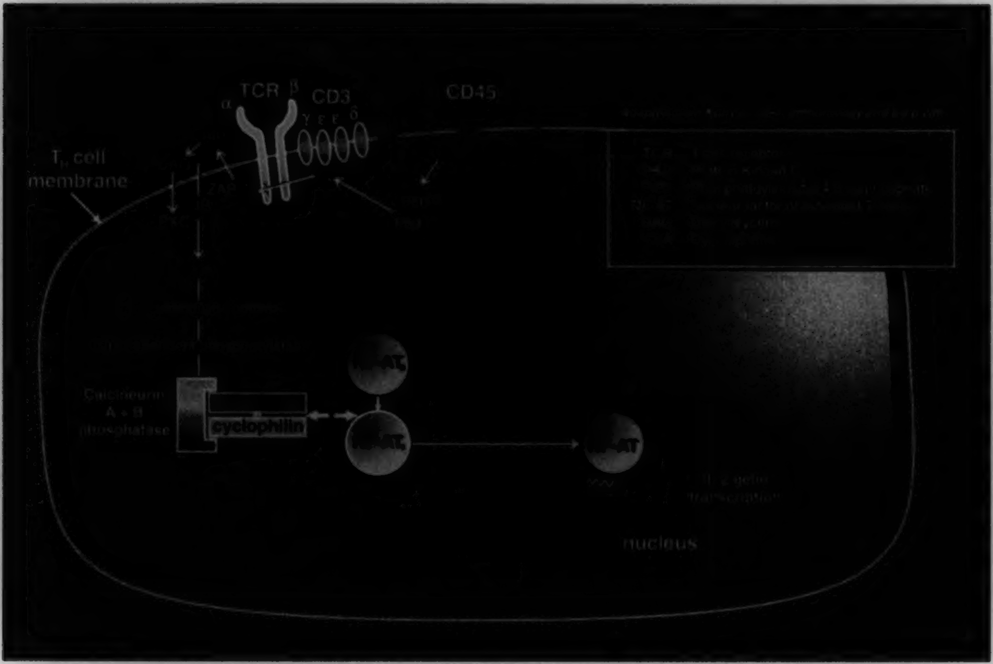

α
TCR
β
CD3
γ ε ε δ
CD45
T_H cell membrane
ZAP
Calcineurin A + B phosphatase
cyclophilin
nucleus

Figure 2.2: Effects of Cyclosporine on the T cell
(Adapted from Kuby J, 1994: Immunology 2nd Ed, p 286)

A grasp of the detailed mechanism of action of CsA has provided us with a richer understanding of a series of molecular signaling events; CsA enters the cytoplasm of T-cells and binds to an immunophilin protein called *cyclophilin* (peptidyl-prolyl cis-trans isomerase).

In turn, the cyclosporine-*cyclophilin* complex binds to a Ca^{2+}-activated serine-threonine phosphatase complex, *calcineurin* (consisting of interactive A and B sub-units), inhibiting its activity(*54, 55*).

This prevents activated *calcineurin* from dephosphorylating the cytosolic transcription factor *NF-ATc* (nuclear factor of activated T-cells–cytosolic), which normally migrates to the nucleus, combines with its nuclear counterpart to form the active *NF-AT* complex (AP-1) required for transcription of the IL-2 gene(*56, 57*). CsA may also inhibit a number of other "early" T cell activation genes (IL-3, GM-CSF, TNF-alpha, c-myc, IFN-gamma).

In the clinic CsA was previously begun using an intravenous formulation (at a dose of 1-2mg/kg/day), and converted to an oral form (at 3 times the IV dose /24h, split q12h) to maintain 12-hour trough CsA levels within the target range outlined above. On account of the lipophilic nature of standard CsA, the bioavailability of the oral form ranged from 10% to 57%(*58*), and may improve over time in any individual. The original CsA formulation has since been replaced by a micro-emulsion formulation called Neoral™. This has an improved absorption profile, and is more bile-independent in its absorption, obviates a need for intravenous therapy, and as a consequence of improved pharmacokinetic profiles has led to lower rejection rates(*59, 60*). Neoral™ may have some advantage over the previous formulation in diabetic patients, where gastroparesis or neuropathic small bowel dysmotility may impair absorption of the previous formulation.

Improved pharmacokinetic monitoring of Neoral™ with two-hour post dose C2-monitoring rather than 12-hour trough level monitoring has led to improved area-under-the-curve of drug exposure, with significantly lower rates of acute rejection in liver and kidney transplantation(*61-64*).

CsA is metabolized by the cytochrome P-450 microsomal enzyme pathway, and is thus subject to the inductive or inhibitory effects of other drugs on this system; appropriate adjustments must be made in CsA dose if interacting drugs are given. The anti-fungal agent, ketoconazole, markedly inhibits CsA metabolism, and the dose of CsA may need to be reduced by >80% to prevent toxicity. Erythromycin has profound effects on CsA metabolism, and should be avoided. The predominant toxic side effect of cyclosporine is nephrotoxicity due to its potent vasospastic effect on the afferent arteriole – an effect which correlates strongly with CsA levels; the calcium channel blockers verapamil, diltiazem and nicardipine all increase CsA levels to a moderate extent, and may be used to advantage both to spare CsA dose and to minimize renal vasospasm. Other nephrotoxic agents may compound the toxicity of CsA–the aminoglycoside gentamicin and all non-steroidal anti-inflammatory agents should be avoided.

Other side effects of CsA include hypertension, diabetes, tremor, headache, seizures rarely (risk increased by derangements in magnesium or cholesterol metabolism), hirsutism, and gum hypertrophy in children. In the recent two years, many patients have been converted from CsA to tacrolimus as although both agents have similar nephrotoxicity profiles; tacrolimus has been associated with lower incidence of hypertension, hyperlipidemia, hirsutism and gingival hyperplasia(*65*).

2.6.1 ADVERSE EFFECTS OF CYCLOSPORINE ON ISLET FUNCTION

The diabetogenic potential of CsA was reported initially by Gunnarsson *et al.* in 1984, in a review of human pancreas/kidney transplant recipients converted from azathioprine-prednisone to CsA-prednisone; peripheral insulin resistance was thought to be the underlying defect(*66*). *In vitro* studies of mouse(*67*), rat(*68*) and human islets(*69*) exposed to extremely high doses of CsA (by current standards) showed deleterious effects on beta-cell function (as reflected by impaired glucose-stimulated insulin synthesis on perifusion or reduced islet insulin content). Further *in vivo* confirmation of CsA induced beta-cell toxicity was obtained in normal rats that became hyperglycemic after three weeks of oral CsA therapy (dose range 15 and 50mg/kg/day), and these changes were reversible after discontinuation of therapy(*70, 71*). Eun *et al.* found that 7 days of CsA treatment (40 mg/kg/day) caused reversible hyperglycemia and hypoinsulinemia in rats(*72*); histological evaluation of the pancreas revealed b-cell degranulation, vacuolization and disrupted endoplasmic reticulum within the cytoplasm. Islets isolated from CsA treated rats had a 50% reduction in mRNA synthesis; these defects were reversible after discontinuation of CsA.

Using in vivo fluorescence microscopy, Rooth *et al.* studied the impact of CsA (15mg/kg) on vascular engraftment of islets transplanted beneath the kidney capsule of mice–and found a marked decrease in neovascularization compared with controls by two weeks; treatment with verapamil prevented any detrimental change by abolishing CsA-induced renal cortical ischemia(*9*). These studies are elegant, but cannot be extrapolated to the clinical situation, where intraportal embolization to the liver has become the favored site; the impact of CsA on portal vasculature is essentially unknown, but is unlikely to parallel that of the renal afferent arteriole.

The interpretation of large animal and human data regarding CsA beta-cell toxicity is often difficult–confounding factors being therapeutic dose that must reflect current practice in terms of target trough CsA level, and attempts to determine the relative contributions of CsA in a multi-drug regimen of azathioprine and steroids where synergism in the causation of diabetes is evident. In dogs, Garvin *et al.* found progressive, reversible dose-dependent impairment of insulin secretion in normal dogs given IV CsA (2-6mg/kg) or oral CsA (12.5mg/kg)(*73*). Alejandro *et al.* found that short-term administration of high-dose CsA to normal beagles (20mg/kg/day–with CsA trough levels maintained at ≥ 500 ng/ml in serum RIA, corresponding to levels of 1000-1200 ng/ml by HPLC(*74*)) caused transient impairment in glucose clearance that returned to normal by one month, but there were permanent defects in insulin, C-peptide and glucagon release, which did not recover at 4 months after discontinuation of therapy(*75*). Wahlstrom *et al.*, using lower CsA dosage in dogs (15mg/kg/day) showed that defects in insulin release were reversible after withdrawal of therapy, the time taken for recovery corresponding to the duration of the CsA treatment course(*76*).

Kneteman *et al.* performed euglycemic clamp studies in dogs bearing intrasplenic islet autografts after 4 months of intramuscular CsA (trough levels adjusted to 500 ng/ml by HPLC)(*74*). Previous studies showed this dose to be effective in preventing rejection of islet allografts in outbred dogs(*36, 37, 77*). While there was baseline impairment of glucose disposal in the pre-treated control dogs resulting from sub-optimal islet mass(*78*), there were no demonstrable additive defects of glucose disposal, insulin secretion or insulin sensitivity due to CsA treatment.

These findings have recently been substantiated in chronic canine intrasplenic islet autograft recipients evaluated by modified Bergman minimal model kinetics on frequently sampled glucose tolerance tests – no significant change in glucose tolerance, fasting or peak insulin, insulin sensitivity or clearance was evident after 30 days of therapeutic range CsA (*74, 79*). It is not clear whether islets embolized intraportally, the favored clinical site, would be exposed to greater potential toxicity from high portal CsA peaks after oral absorption.

Discrepancies between the studies outlined above are accounted for largely by differences in therapeutic dose of CsA; target trough levels of CsA used in current clinical practice rarely exceed 300-350ng/ml by HPLC early after transplantation, and are typically considerably lower in long-term stable recipients (150-175ng/ml). CsA monotherapy is not used routinely in the early post transplant period in man however, since the therapeutic index for nephrotoxicity in patients is far narrower compared to the dog; attempts to maintain long-term stable pediatric liver transplant recipients on CsA monotherapy have met with success–the incidence of rejection was 11% at 1 year after azathioprine and steroid withdrawal, the major predisposing factor to late rejection being sub-therapeutic CsA levels(*80*).

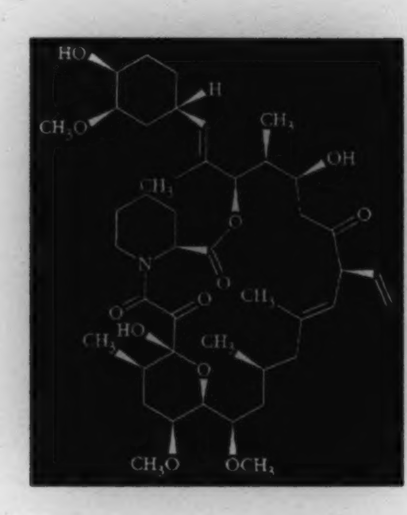
In patients receiving CsA as part of triple immunosuppression (with steroids and azathioprine) the incidence of post transplant diabetes varies between 4% and 20%, and of these 40% will require insulin therapy(*6, 81*). Boudreaux *et al.* found an increased incidence of post transplant diabetes in renal transplant recipients randomized to treatment with CsA-prednisone-azathioprine ± induction ALG compared with patients receiving only azathioprine-prednisone, despite a reduction in steroid dose(*82*).

In a double blind, randomized study of 13 patients with multiple sclerosis treated with CsA monotherapy or placebo for two years, no adverse impact of conventional dose CsA (4-18mg/kg) was seen on any parameter of insulin output or glucose clearance after arginine potentiated glucose stimulation(*83*). A subsequent study from the same group showed defective b-cell function by arginine-potentiated glucose stimulation in psoriasis patients treated with CsA, but not in arthritis patients receiving long-term steroids; pancreas transplant recipients treated with CsA-prednisone-azathioprine had significantly less insulin secretion(*84*).

Thus, the diabetogenic potential of CsA monotherapy at supra-therapeutic dose in large animals and man is clear, as is the synergistic interaction of CsA with steroids and azathioprine when used in combination at therapeutic dose in man. The diabetogenic potential of CsA monotherapy is not clearly established when given at a dose that reflects current practice in transplantation (12h trough levels of 150-350ng/ml HPLC) – an important consideration for islet transplantation if CsA is to be used in combination with some of the newer, more potent, immunosuppressants with no demonstrated diabetogenic potential (such as rapamycin or mycophenolate mofetil), that may eventually replace the steroid and azathioprine components of therapy.

Reviewing the results of islet transplantation maintained by the Islet Transplant Registry(*3*), despite the adverse metabolic impact of CsA and steroids(*85*), insulin independence has been achieved for periods ranging from 3 months to 4 years in patients(*3, 8, 86, 87*). Furthermore, there are currently islet allograft recipients with ongoing C-peptide secretion beyond 9 years post transplant(*3, 88*).

2.7 TACROLIMUS



Tacrolimus (FK506) is a macrolide antibiotic isolated from the soil fungus *Streptomyces tsukubaensis*, and first brought to light by Ochiai and colleagues in 1987 for its potent immunosuppressive properties(*89, 90*). While structurally unrelated to CsA, it shares with CsA many of the same intra-cytoplasmic pathways in the blockade of IL-2 production. Tacrolimus appears to have a broader specificity of action than CsA, through suppression of proliferation of activated B cells, affecting both G0-to-G1 and G1-to-S phases of the cell cycle(*91*).

The potency of tacrolimus *in vitro* was shown to be 10-100 times that of CsA by inhibition of mixed lymphocyte culture and the generation of cytotoxic T cells(*92*). *In vivo*, FK-506 prevented allograft rejection of skin(*93*), liver(*94*)and heart(*90*) in rats, and of kidney transplants in dogs(*89*) and baboons(*95*). These studies paved the way for its first use in human liver transplantation by Starzl *et al.* in 1989, who showed that grafts with refractory rejection could be rescued by conversion from CsA to FK-506(*45*). A single-centre randomized trial of tacrolimus vs. CsA in liver transplantation from

Pittsburgh reported a reduced incidence of acute rejection, and showed that steroid dose could be reduced dramatically(*96*).

One year follow-up data from two large multi-centre randomized trials of FK506 vs. CsA in liver transplantation were reported from centres in Europe(*97*) and the United States(*65*). No difference in patient or graft survival was evident; a modest (<10%) but statistically significant reduction in incidence of acute rejection was reported in the tacrolimus treatment arm in both trials. The incidence of chronic and refractory rejection was lower in tacrolimus treated patients, and was reflected in a reduced need for OKT3 treatment. These benefits were offset by an increased incidence of nephrotoxicity, neurotoxicity and hyperglycemia in the tacrolimus group; CMV infections and pneumonia were less common however, and hirsutism and gingival hyperplasia were not seen in tacrolimus treated patients. Five year follow-up data is now available on the initial cohort patients in these multicentre trials, and clearly show benefit for tacrolimus, with significantly enhanced liver graft and patient survivals compared with cyclosporine, leading to a prolonged graft half-life(*98*). The ability of tacrolimus to reverse refractory rejection represents a major benefit for those patients who would otherwise die without access to urgent re-transplantation(*99, 100*).

Tacrolimus is typically given at a starting dose of 0.05mg/kg PO twice daily – less than one quarter of how this drug was used initially, when significant side effects were noted in early clinical studies. Levels are then controlled based on trough drug level concentration.

Dose may be titrated primarily against renal function, and trough tacrolimus levels used as a secondary guide (target range 10-15ng/ml initially, reducing to 5-10ng/ml or lower in maintenance). The above dosage recommendations are usually doubled in the pediatric population, since tacrolimus is less well absorbed in children.

The IV formulation is not recommended (unless there are major difficulties with oral absorption) since the IV route was associated with a high incidence of side effects in the initial phase of the U.S. multi-centre trial(*65*). It is recommended that CsA be discontinued for 24 hours prior to conversion to tacrolimus; both agents should not be used concurrently in view of the high risk of additive side effects. The tacrolimus drug interaction profile is similar to CsA, since both are metabolized through the cytochrome P-450 pathway.

The mechanism of action of tacrolimus is almost identical to CsA, except that it binds to a different cytoplasmic immunophilin called *FK-binding protein* (FKBP). Both CsA-cyclophilin and FK-506–FKBP12 drug-immunophilin complexes binds to a common target, *calcineurin*, preventing dephosphorylation of NF-ATc, and thus assembly of a functional transcription factor essential for nuclear IL-2 gene transcription(*54*).

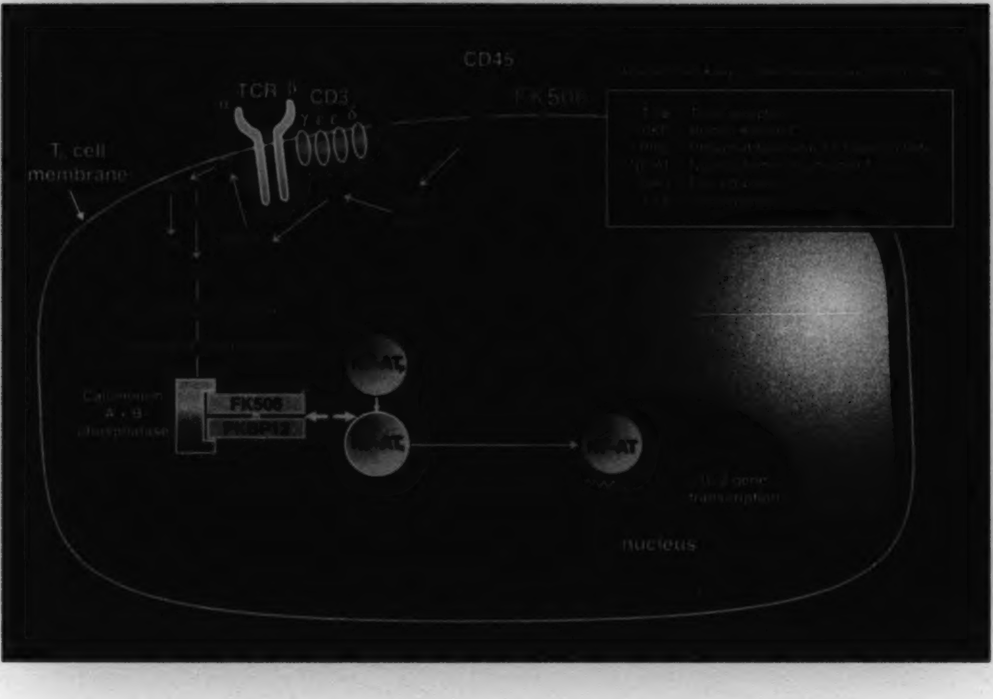

CD45
TCR
α
β
CD3
γ ε ε δ
FK506
T_h cell
membrane
Calcineurin
A + B
phosphatase
FK506
FKBP12
NF-AT
IL-2 gene
transcription
nucleus

Figure 2.3: Effects of Tacrolimus (FK506) on T cell function

Griffith *et al.* demonstrated most of these intermolecular interactions by elaborate x-ray crystallography with three-dimensional reconstruction(*101*). The interaction of a groove in calcineurin A (calcineurin B-binding a-helix (BBH)) with calcineurin B, the structure of the tacrolimus – FKBP12 complex, and the binding of the tacrolimus – FKBP12 complex to calcineurin at the base of the BBH groove have been visualized clearly. The remarkable finding is that tacrolimus – FKBP12 does not in fact bind directly to the active phosphatase site on calcineurin A – rather it blocks access of NF-ATc to that active phosphatase domain through allosteric hindrance.

Figure 2.4: Interaction of tacrolimus with FKBP12 – crystal structures and molecular interactions *(after Griffiths et al, Cell 1995: 82; 507-522, with kind permission)*

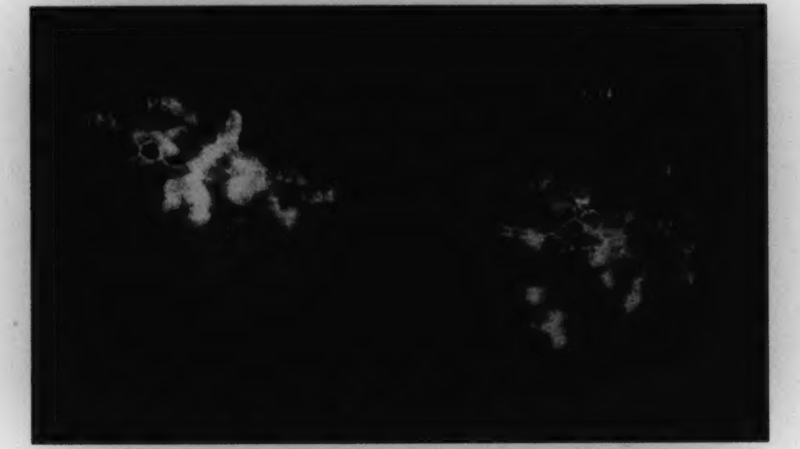


Figure 2.4 (a): X-ray crystal structure of the FKBP12-FK506 complex with calcineurin

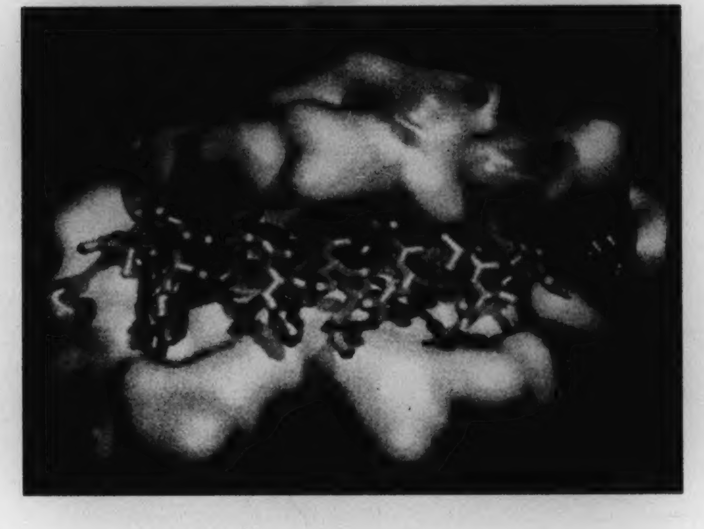


Figure 2.4 (b): Molecular interactions of the Calcineurin B binding alpha helix of Calcineurin A (space-filling model) with calcineurin B (electrostatic model)

Figure 2.4 (c): X-ray crystal structure of the FKBP12-FK506 complex with calcineurin. Calcineurin A (blue), Calcineurin B (green), FK506 (while), FKBP12 (red), phosphatase binding site on calcineurin A (yellow)

Figure 2.4 (d): Electrostatic model of FK506 interaction with FKBP12. The binding of FK506-FKBP12 to Calcineurin A/B blocks access to the phosphatase active site on Calcineurin A (yellow), which would otherwise dephosphorylate NFAT and lead to IL-2 transcription.

2.7.1 ADVERSE EFFECTS OF TACROLIMUS ON ISLET FUNCTION

Dose-dependent diabetogenic effects of FK-506 were first noted in baboons(*102*) and in cynomologus monkeys after pancreas transplantation(*103*). Todo *et al.* reported similar findings subsequently in man(*95*). *In vitro*, FK-506 has been noted to inhibit insulin secretion from rat islets in culture, but only at higher concentrations (10-100ng/ml) than required to suppress the immune response (5ng/ml)(*104*). Hirano *et al.* found glucose intolerance and islet vacuolization of rat islets when FK-506 was given at extremely high dose (10mg/kg/day) for two weeks(*105*). Tze *et al* were unable to show negative impact of therapeutic dose tacrolimus (0.1-1.0 nM/L) on either rat or human islets in culture(*106*). Isolated canine islets in culture failed to show impairment of first or second phase insulin release on stimulation after exposure to concentrations of FK-506 up to 100 nM, whereas the converse was found for CsA exposed islets(*107*). Human islets transplanted into nude mice showed no evidence of impaired glucose clearance after 7 days of FK-506 at a dose of 0.3 mg/kg/day; doses above 1 mg/kg/day induced significant impairment in graft function(*108*).

Tamura *et al.* found that insulin production was impaired at the transcription step by tacrolimus in *in vitro* insulinoma cell lines, and interestingly, the beta-cell content of FKBP-12 was high, suggesting that the tacrolimus – FKBP12–calcineurin complex within the b-cell might interfere with nuclear transcription factors for insulin(*109*). Ricordi *et al* showed *in vivo* that physiological concentrations of tacrolimus did not influence the function of human islets transplanted into diabetic nude mice, but higher doses impaired glucose tolerance and blunted C-peptide responsiveness(*110*)

In a study of normal beagle dogs treated with 1 mg/kg/day tacrolimus orally for one month, defects in insulin secretion and glucose utilization were observed; these changes were mostly reversible, although insulin secretion failed to recover to baseline in follow-up(*111*).

In hyperglycemic clamp studies of patients receiving therapeutic level FK-506 monotherapy for psoriasis, multiple sclerosis or primary biliary cirrhosis (FK-506 levels 0.1-1.0 ng/ml) Strumph *et al.* noted lower steady state insulin secretion and a trend towards lower peak first phase insulin secretion(*112*). Jindal *et al.*, in a prospective study of liver transplant patients treated with FK-506 and low-dose steroids vs. CsA and high-dose steroids, found no difference in the incidence of either temporary or permanent diabetes between CsA and tacrolimus(*113*). Tacrolimus has been used successfully in pancreas transplantation without any evidence of adverse impact on glycemic or lipid metabolism(*114, 115*). Indeed the current significant improvement in whole pancreas transplant outcomes has been largely attributed to the use of tacrolimus together with mycophenolate(*116-118*).

Tacrolimus high-dose monotherapy has proved to be highly successful in non-diabetic patients undergoing combined islet-liver transplantation after abdominal exenteration – of 11 patients, 55% were independent of insulin for periods ranging from 5-58 months post transplant, death from recurrent disease being the cause of graft loss(*42, 119*). The use of tacrolimus in combination with glucocorticoids only rarely has resulted in even temporary insulin independence in type 1 diabetic patients undergoing islet transplantation(*85, 120*).

Significant local damage to transplanted islets contained within whole pancreas transplants has been observed in association with high dose tacrolimus therapy, leading to vacuolization, apoptosis or fibrosis of human islets transplanted in patients(*121*).

In summary, therefore, it is now apparent that tacrolimus is more potent than cyclosporine in controlling both autoimmune recurrence and acute rejection after whole pancreas transplantation, and the avoidance of cyclosporine-mediated gum hypertrophy makes this an attractive drug to use at low dose in combination with other less diabetogenic therapies after islet transplantation. Studies are proposed in the current thesis to explore this option further, synergizing the immunologic benefits of tacrolimus while minimizing diabetogenic toxicity.

2.8 MYCOPHENOLATE MOFETIL (CELLCEPT)

Mycophenolic acid (MPA) is a fermentation product of several *Penicillium* fungal species, and was first studied by Japanese investigators in 1969 for anti-tumor activity(*122, 123*). Although immunosuppressive side effects were noted in the initial studies, the true potential of this compound was not recognized until 1982, when Allison *et al.* was searching for specific inhibitors of the *de novo* synthesis of guanosine monophosphate (GMP)(*124-126*). MPA strongly inhibited the human lymphocyte response to mitogenic stimuli, and inhibited both humoral and cell mediated immune responses in mice(*125*).

MPA was first applied clinically in the treatment of psoriasis, and appeared promising initially. Widespread clinical trials were discontinued in 1977, however, after fear of MPA's immunosuppressive and carcinogenic potentials became apparent, in addition to acute gastrointestinal side effects noted in some patients. A report by Epinette *et al.* of 85 psoriasis patients receiving oral MPA (2-7 g/day) on compassionate basis for up to 13 years, suggested that gastrointestinal side effects became more infrequent with time, and that the agent was well tolerated; 7% of these patients developed skin cancers or other tumors, however(*127*).

Mycophenolate mofetil (MMF) (RS 61443) is the morpholinoethyl ester of MPA, and was found to double the bioavailability of MPA in primates(*128*). MMF is a pro-drug, and once absorbed, is hydrolyzed rapidly by plasma and tissue esterases to yield MPA(*129*). MPA is deactivated by conjugation to beta-glucuronide in the liver, and the inactive glucuronide conjugate accounts for up to 90% of the total circulating drug *in vivo*. An extensive entero-hepatic recirculation of MPA-glucuronide effectively prolongs the half-life of active MPA, since glucuronyltransferases and beta-glucuronidases lead to interconversion back to MPA(*128*).

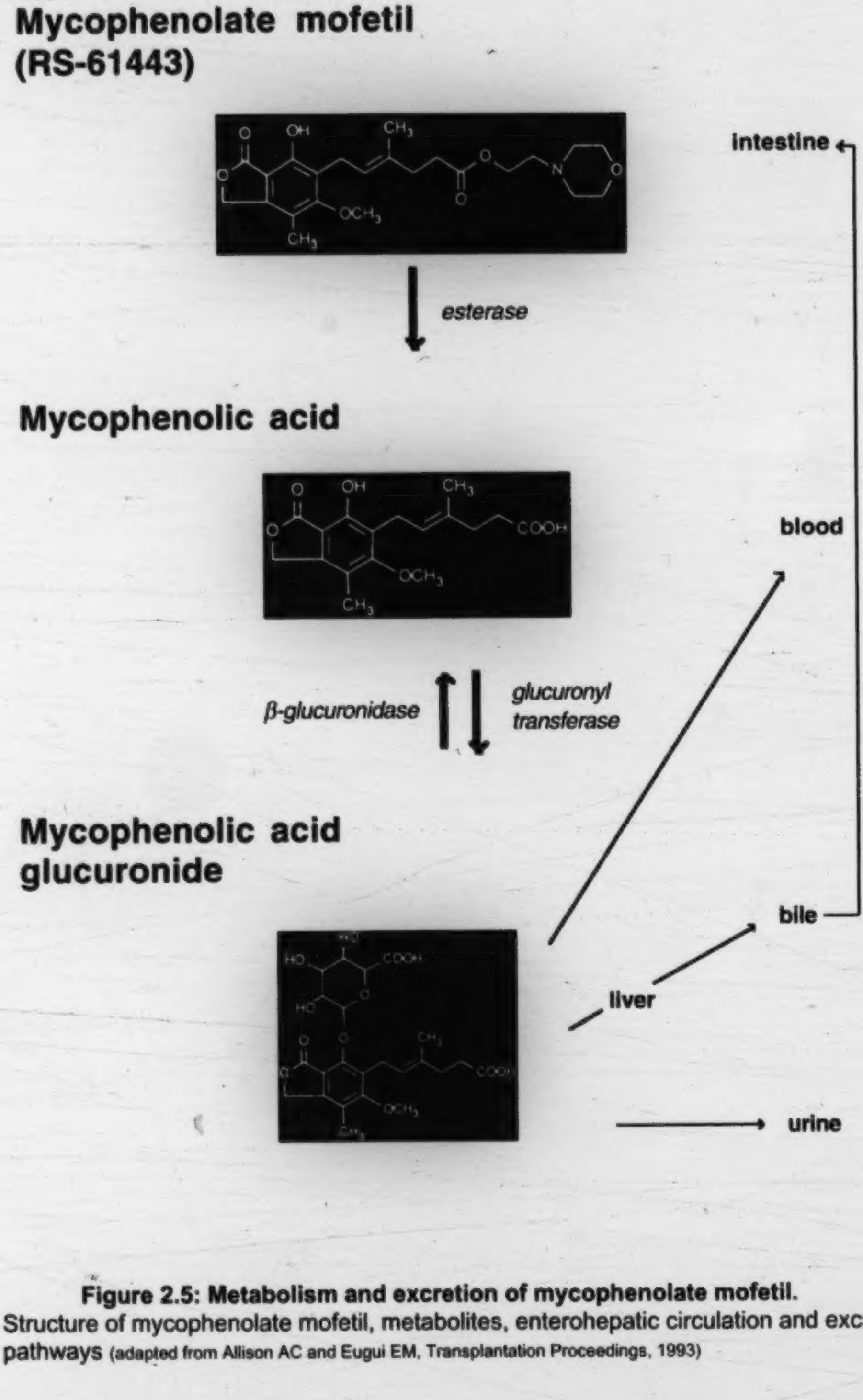
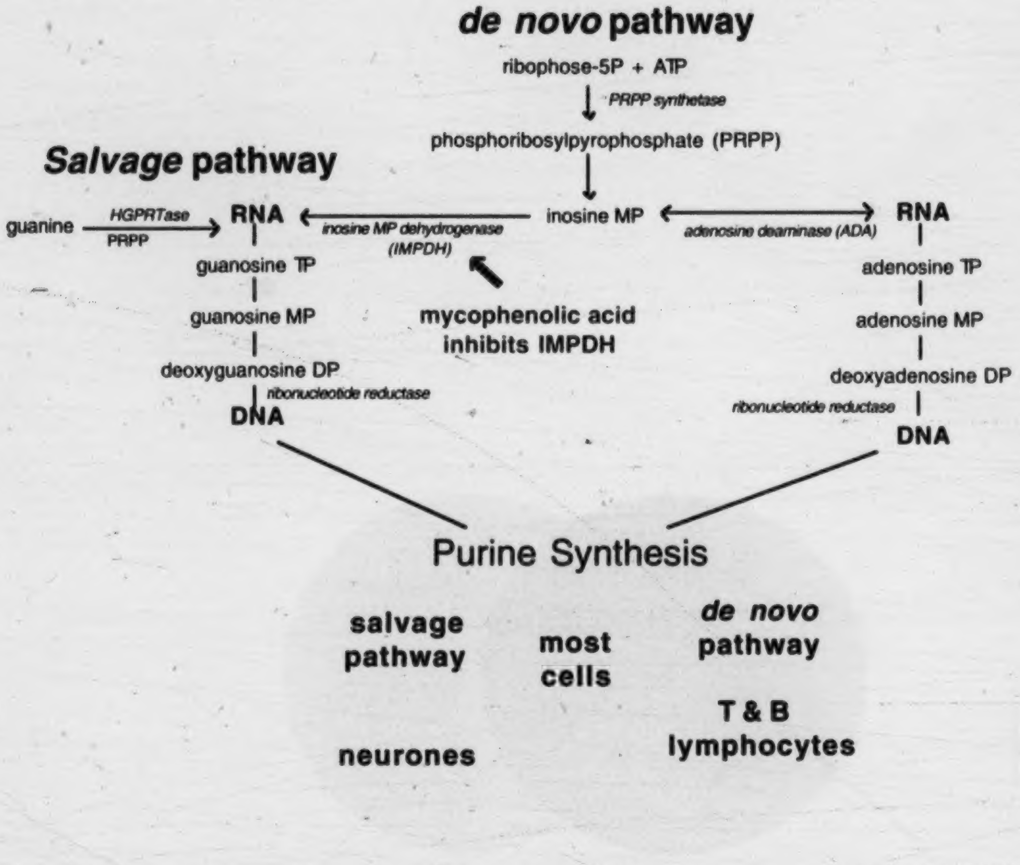

Mycophenolate mofetil
(RS-61443)
esterase
Mycophenolic acid
β-glucuronidase
glucuronyl transferase
Mycophenolic acid glucuronide
intestine
blood
bile
liver
urine

Figure 2.5: Metabolism and excretion of mycophenolate mofetil.
Structure of mycophenolate mofetil, metabolites, enterohepatic circulation and excretion pathways (adapted from Allison AC and Eugui EM, Transplantation Proceedings, 1993)


de novo pathway
ribophose-5P + ATP
PRPP synthetase
phosphoribosylpyrophosphate (PRPP)
Salvage pathway
guanine
HGPRTase
PRPP
RNA
inosine MP dehydrogenase (IMPDH)
inosine MP
adenosine deaminase (ADA)
RNA
guanosine TP
guanosine MP
deoxyguanosine DP
ribonucleotide reductase
DNA
mycophenolic acid inhibits IMPDH
adenosine TP
adenosine MP
deoxyadenosine DP
ribonucleotide reductase
DNA
Purine Synthesis
salvage pathway
most cells
de novo pathway
neurones
T & B lymphocytes

[Adapted from Allison A.C and Eugui E.M., Transplantation Proceedings 1993, and from Groth C. Transplantation Proceedings 1995]

Figure 2.6: Mycophenolate mofetil (Cellcept) – mechanism of action
Mycophenolate mofetil inhibits IMPDH activity, thereby preventing de novo synthesis of purine nucleotides. Specific inhibition of lymphocyte proliferation occurs, with minimal effect on other cell types, since lymphocytes are unique in lacking salvage pathways.

The immunosuppressive actions of MPA result from potent but reversible inhibition of IMP dehydrogenase (IMPDH) activity, a key enzyme in the *de novo* synthesis of guanosine nucleotides. T and B-lymphocytes rely solely on *de novo* synthesis of purines, and MPA effectively blocks their proliferative activity – T helper response, antibody production, and the generation of cytotoxic T cells are therefore impaired(*130*). Most other cells possess an alternative *salvage* pathway for purine synthesis, and are thus relatively spared from MPA toxicity(*131*). Allison *et al.* have further shown that MPA inhibits the glycosylation of adhesion molecules and the binding of human lymphocytes to activated endothelial cells, by guanosine nucleotide depletion(*131*). This may explain the efficacy of MPA in the treatment of established rejection, after clonal expansion of anti-donor lymphocytes has occurred.

Human IMPDH exists in two distinct isoforms(*132*). Type I IMPDH is constitutively expressed on resting cells; type II IMPDH is inducible in rapidly dividing cells, particularly in activated human T and B-lymphocytes. Modulation in overall IMPDH expression appears to result from changes in synthesis of the type II isoform. MPA inhibits the type II isoform with five-fold more affinity than type I, and type II isoform inhibition in lymphocytes appears to be the major target of MPA(*125, 133*). The human gene for type II IMPDH has been mapped to a 12.5 kb region on chromosome 3(*134*). Pharmacodynamic monitoring of inhibition of IMPDH activity has been shown to be a more effective means of assessing immunosuppressive efficacy than MPA levels alone(*135, 136*). Morris *et al.* showed that MMF monotherapy prevented rejection of cardiac allografts in rats, and could induce a state of donor-specific tolerance(*130*). Hao *et al.* found that oral MMF (80 mg/kg/day) for 30 days prevented rejection of islet allografts in streptozotocin-diabetic mice and rats, and induced indefinite graft survival in 64% of recipient mice(*137*). Combination treatment with CsA for 30 days improved indefinite survival to 89%, but the tolerance induced was less stable since more long-term surviving grafts were lost after challenge inoculation with donor strain splenic cells(*137*).

Platz *et al.* found that MMF monotherapy (40 mg/kg/day) prolonged kidney allograft survival in dogs to a median of 34 days, but was associated with marked gastrointestinal side effects (gastritis, diarrhea, and anorexia)(*138*); A lower dose of MMF (20 mg/kg/day), in combination with CsA (5 mg/kg/day) and prednisone (0.1 mg/kg/day) was highly effective in preventing renal allograft rejection, and was better tolerated in the dog. The limiting gastrointestinal side effects of MMF in the dog are not observed to the same extent in rodents, monkeys(*128, 139*). Canine gut epithelium has much lower IMPDH activity than other species, and may account for the increased toxicity(*138*). Similar gastrointestinal toxicities have been reported in a large randomized controlled comparison of MMF with azathioprine in clinical liver transplantation (n=565), where 8% of patients in the MMF arm discontinued treatment because of gastrointestinal side effects(*140*).

Platz and colleagues showed subsequently that MMF was highly effective in reversing established renal allograft rejection in 87% of dogs when given at very high dose (80 mg/kg/q12h for 3 days), but it took up to 3 weeks to restore creatinine levels to normal(*138*). Interestingly this three-day very high dose regimen was tolerated without side effects. MMF was shown subsequently to prolong liver and small bowel allograft survival in dogs (20 mg/kg/day), when given in combination with CsA and low dose steroids(*141, 142*).

Sollinger *et al* reported efficacy of MMF in the treatment of refractory renal allograft rejection that failed to respond to high dose steroids or OKT3(*143*); of 75 patients given MMF 1.0 - 1.5 g/q12h, an impressive 69% achieved successful long-term graft rescue. The major side effects were gastrointestinal, and were mostly self-limiting, or responsive to dose reduction or divided day dosage. The infection rate was high (40%), but in keeping with expectation for a patient group heavily immunosuppressed with sequential high-dose steroid and OKT3 before MMF. No evidence of nephrotoxicity, hepatotoxicity or bone marrow suppression was observed.

Two large randomized double blind controlled trials assessing efficacy of MMF in primary renal allograft recipients were reported subsequently. The U.S. multi-centre trial enrolled 495 patients randomized to receive MMF at 1.0 g q12h, 1.5 g q12h (irrespective of body weight) or azathioprine 1-2 mg/kg/day, in addition to CsA and steroids(*15*). The incidence of first biopsy-proven rejection was reduced by half in patients treated with MMF compared to azathioprine (19.8% (MMF 2 g/day), 17.5% (MMF 3 g/day), 38% (azathioprine), but there was no difference in graft or patient survival at six months. The higher dose MMF (3 g/day) was marginally more effective in reducing rejection, but was associated with a higher incidence of diarrhea, leukopenia and CMV disease.

The European multicentre MMF trial compared MMF (2 g or 3 g per day) with placebo, and enrolled 491 patients(*144*). Significantly fewer patients developed biopsy-proven rejection when given MMF 3 g (17%), 2 g (14%), compared with placebo (46%). A modest increase in gastrointestinal side effects was noted in the MMF groups, and was dose-related (2 g 45%; 3 g 53% vs. placebo (42%). The frequency of tissue-invasive CMV disease and herpes infection, and leukopenia was highest in the MMF 3 g treatment group. Patient and graft survival data were comparable in all groups (95% and 90% respectively). The requirements for corticosteroid and antilymphocyte agents for acute rejection was significantly lower in the MMF groups. No difference in risk of malignancy was evident. The incidence of tissue-invasive CMV infection and gastrointestinal side effects were higher in patients receiving 3g per day dosage(*145*). Three-year follow-up data was reported in 1999, with a 7.6% reduction in the incidence of graft loss in MMF-treated recipients, confirming that the early effects in reduced incidence of acute rejection translated into a reduced rate of late graft loss(*146*). While these findings are encouraging, longer-term data is required before it is known what influence MMF may have in enhancing renal allograft half-life through a reduced risk of chronic allograft nephropathy.

2.8.1 EFFECT OF MYCOPHENOLATE ON ISLET GRAFT FUNCTION

Sandberg *et al.* found that isolated rat islets exposed to MMF (dose 15-240 mg/L) for 6 days in culture had reduced DNA content, with derangement in islet architecture at the highest doses, glucose-stimulated insulin release was reduced by 75-90%, but pro-insulin biosynthesis was unaffected(*147*). These effects were reversible on withdrawal of the drug. *In vivo* studies in rats and mice given large doses of MMF (70 mg/kg i.p.) showed only minor defects in glucose tolerance, and no difference in pancreatic insulin content(*147, 148*). The relevance of *in vitro* studies of a pro-drug (MMF) that is converted rapidly in vivo to MPA is unclear. Islets exposed to MPA in guanine-free media in culture showed inhibition of glucose-stimulated insulin release by 68% at a dose of 50 micrograms/ml(*149*). Guanine-rich media did not affect islet function after addition of MPA, showing that while both de novo and salvage pathways of purine nucleotide biosynthesis are present in rat islets, the salvage pathway provides the major source of intra-islet nucleotides(*150*).

Studies of chronic MMF administration to long-term canine islet autografts (20 mg/kg/day) revealed a 20% decline in glucose clearance during treatment with MMF alone or in combination with CsA – changes which recovered completely after discontinuation of therapy (*79*). These effects were largely accounted for by decreased total insulin release after glucose stimulation, since no impact on insulin sensitivity was seen.

There was no difference in incidence of post transplant diabetes seen in any of the large multicentre MMF trials outlined above. It is difficult to predict what overall benefit this agent might offer for clinical islet transplantation, since a modest reduction in rate of acute rejection may be partially offset by impairment of metabolic function, if used in combination with calcineurin inhibitors. One possibility may be to use mycophenolate in combination with sirolimus. In sparing overall dose requirements for both steroids and CsA, any adverse impact of MMF may be less pronounced.

The substitution of tacrolimus with Cellcept raises some potential concerns, namely:

a) Will combined sirolimus/Cellcept have similar immunologic potency to sirolimus/tacrolimus in prevention of autoimmune recurrence and acute rejection after islet transplantation?

b) Side effect profiles for sirolimus and Cellcept potentially overlap since both agents may cause neutropenia – will this be exacerbated when the two agents are combined?

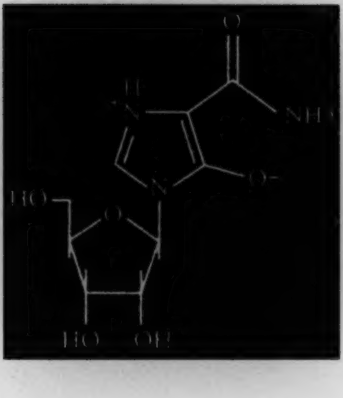
Pre-clinical data has shown that concomitant therapy of sirolimus + Cellcept produced synergistic effect in prevention of pancreas, kidney and heart allograft rejection, and in reversal of established rejection(*151*). Compelling evidence from clinical trials now suggest that sirolimus + Cellcept may be safe and effective if applied to islet transplantation, but only under circumstances where sirolimus dosing is adjusted according to target level:

a) A series of 53 patients were converted to sirolimus/Cellcept from tacrolimus/Cellcept six months after kidney and kidney/pancreas transplantation at the University of Memphis – importantly, the incidence of acute rejection was less than 4%, there was no recurrence of autoimmunity, and although total white cell counts were on the lower side of the normal range, there were no cases of clinically significant neutropenia, thrombocytopenia or PTLD(*152*). These patients experienced improvement in renal allograft function and had better control of hypertension(*152*). It is acknowledged that immunosuppressant conversion protocols may have different outcome than *de novo* therapies, however.

b) MMF has been used as part of mainstay immunosuppression in patients undergoing whole pancreas transplantation, and the introduction of MMF has been strongly associated with improvements in pancreas graft survival in autoimmune recipients(*117, 118, 153*).

c) A series of 14 patients with renal function after liver or kidney transplantation were immunosuppressed primarily with sirolimus + Cellcept + daclizumab, and the combination appeared to provide effective non-nephrotoxic immunosuppression without the need for a lymphocyte-depleting regimen(*154, 155*). A 14% acute rejection rate was associated with sub-therapeutic levels of sirolimus in all cases.

A randomized multicenter trial of sirolimus/Cellcept vs. cyclosporine/Cellcept in renal allograft recipients (n=40) demonstrated acceptable safety and efficacy profiles – suggesting that sirolimus/Cellcept could be used as primary therapy to prevent rejection(*156*). The biopsy-proven rejection rate of 27% was of concern – but lower therapeutic doses of sirolimus were given, and daclizumab induction was not given.



2.9 MIZORIBINE

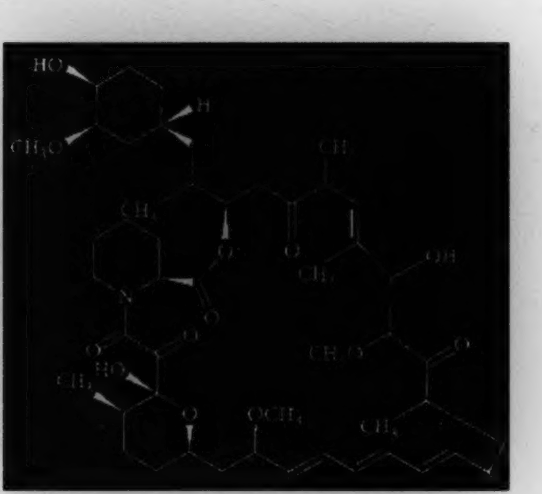
Mizoribine (bredinin) is an imidazole nucleoside isolated from *Eupenicilium brefeldianum* by Mizuno *et al.* in Japan in 1974(*157*). Its mechanism of action is similar to MMF, in that, after phosphorylation, it inhibits IMPDH in the *de novo* pathway of purine synthesis(*149*). It is less lymphocyte selective than MMF however, and has not been investigated in clinical trials outside of Japan.

Unlike azathioprine, this compound is not taken up by nucleic acids in the cell. Instead, after phosphorylation Mizoribine-monophosphate inhibits GMP synthesis by the antagonistic blocking of IMPDH and GMP- synthetase. By reducing stability of cyclin A mRNA in human B cells, mizoribine may further down-regulate their responses(*158*). Mizoribine binding proteins may include 4-3-3 proteins – which are key to downstream cellular signal interactions, stimulating transcriptional activation of the glucocorticoid receptor(*159*).

The drug has been found to inhibit both humoral and cellular immunity, and on this basis it was developed as an immunosuppressant. Median effect analysis revealed evidence of synergism of mizoribine with tacrolimus in enhancing rat heart allograft survival(*160*), and was synergistic with CsA in maintaining dog renal allografts(*161*). Mizoribine has been well tolerated clinically, and has proven effective in preventing renal allograft rejection(*162*), and for the treatment of lupus nephritis, rheumatoid arthritis and the nephrotic syndrome(*163, 164*).

Data is lacking on the effects of mizoribine on islet function, and it has not been evaluated in islet transplantation. With the availability of MMF, it seems unlikely that mizoribine will be explored further at present.

2.10 SIROLIMUS (RAPAMYCIN)



Sirolimus (rapamycin) is a macrolide lactone antifungal fermentation product of *Streptomyces hygroscopicus*, an actinomycete isolated originally from soil samples from the Vai Atore region of Easter Island (Rapa Nui), from where it derives its name(*165, 166*). While the reasons for a soil organism to secrete compounds with immunosuppressive properties are not fully understood, it seems likely that

Streptomyces evolved to secrete sirolimus as a toxin to inhibit the growth of competing yeast and fungi(*167*). The agent was explored initially for its potency against Candida, but it rapidly became apparent that rapamycin was a powerful immunosuppressant in rat models of autoimmune arthritis and encephalomyelitis(*168*).

Interest in sirolimus surged when it was recognized that its molecular structure was similar to tacrolimus. Morris *et al.* were the first to demonstrate the potent efficacy of rapamycin in organ transplantation, in heterotopic heart allografts in mice and rats(*169*). Studies from Cambridge, England, confirmed prolongation of rat heart and pig kidney allografts(*170*). Sirolimus is non- nephrotoxic and does not appear to potentiate nephrotoxicity from cyclosporine(*171*), but has been associated with gastrointestinal side effects secondary to vasculitis in dogs and monkeys(*172*). Other reported side effects at high dose in animals include weight loss, testicular atrophy and lethargy. The early development and pre-clinical evaluation of sirolimus have been reviewed extensively by Kahan and Morris(*139, 173, 174*)

Although sirolimus is a pro-drug, and binds to identical cytosolic binding proteins as FK-506 (FKBP-12 and FKBP-25), the mechanism of action of sirolimus is quite different. It does not cause inhibition of calcineurin, even at high dose, and thus does not interfere with T cell early activation genes. Sirolimus is an antagonist to the immunosuppressive actions of FK-506 *in vitro*, but interference *in vivo* is minimal because clinical therapeutic targets for these drugs do not saturate the binding capacity kinetics of the FKBP binding proteins(*175, 176*) The rapamycin-FKBP-complex is unable to interact sterically with calcineurin–the larger rapamycin active domain would collide with the backbone of calcineurin B, and rapamycin lacks a C21 allyl residue which is a key interactive element for FK-506-calcineurin binding(*101, 177*).

Sirolimus blocks T and B lymphocyte responses to IL-2 and other cytokines by interference with phosphorylation events that would otherwise follow binding of IL-2 to its receptor. Cell-cycle kinetics are thus interrupted in late G1, before entry into the S phase, as late as 14 hours after initial stimulation *in vitro*. Sirolimus is calcium-independent in its activity. By contrast, CsA and FK-506 prevent immune activation at an earlier phase (G0/1 interface), within a few hours of activation *in vitro*, and are calcium-dependent. By impeding cytokine action, T and B cell recruitment, activation and expansion is prevented. The actions of sirolimus are more specific than other anti-proliferative agents, since the drug prevents only growth factor induced mitogenesis, leaving other proliferative pathways intact.

Newer analogues of sirolimus, notably 40-0-(2-hydroxyethyl) rapamycin (RAD) have been developed. RAD is a derivative of rapamycin developed by Novartis with similar efficacy and side effect profile to sirolimus. Early clinical renal and liver transplant experience does not suggest increased potency, and the dyslipidemic side effects are similar to the parent sirolimus compound(*178*). Phase III clinical trials are currently being completed in kidney, heart and liver transplantation(*179-182*). The combination of RAD with FTY720 has shown marked synergism both in rodent and primate renal allograft transplantation(*183, 184*).

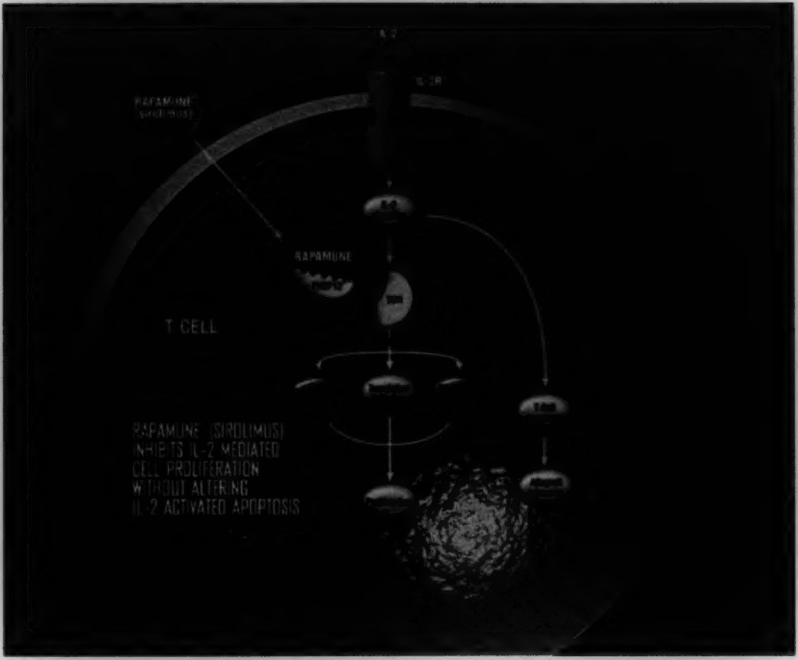

IL-2
IL-2R
RAPAMUNE (sirolimus)
RAPAMUNE
T CELL
RAPAMUNE (SIROLIMUS) INHIBITS IL-2 MEDIATED CELL PROLIFERATION WITHOUT ALTERING IL-2 ACTIVATED APOPTOSIS

Figure 2.7: Binding of sirolimus to FKBP-12 within the T-cell, and subsequent coupling to the mammalian target of rapamycin (mTOR)
(Modified from Dr Suren Sehgal with kind permission)

The molecular target for the sirolimus-FKBP complex is a kinase termed the mammalian target of rapamycin (mTOR); other synonyms include FKBP-rapamycin-associated protein (FRAP/RAFT-1), which has now been cloned(*174, 185, 186*). While the subsequent downstream pathways are multiple, the dominant pathway involves selective inhibition of phosphorylation of p70 S6 tyrosine kinase. The intracellular pathways of sirolimus action have been summarized in recent reviews by Sehgal *et al*(*187, 188*). The relevance of metabolites of sirolimus is not known presently(*189*).

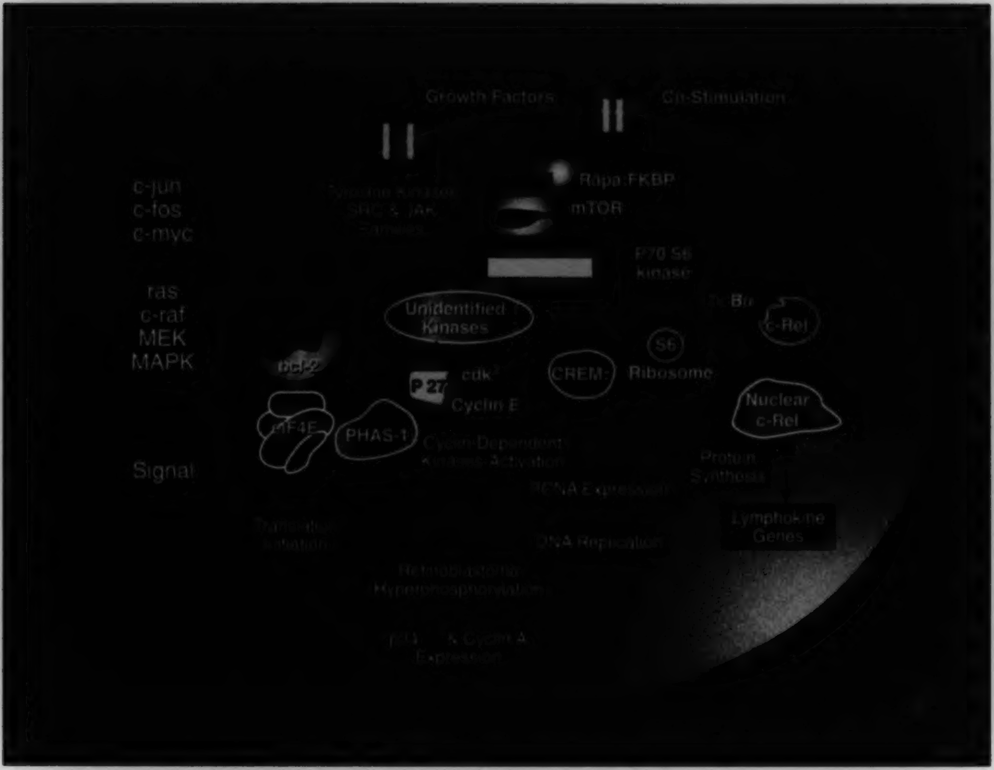

Growth Factors
Co-Stimulation
Rapa:FKBP
mTOR
c-jun
c-fos
c-myc
Tyrosine Kinases
SRC & JAK
Families
P70 S6
kinase
ras
c-raf
MEK
MAPK
Unidentified
Kinases
S6
c-Rel
bcl-2
P 27
cdk2
Cyclin E
CREM
Ribosome
Nuclear
c-Rel
eIF4E
PHAS-1
Cyclin-Dependent
Kinases Activation
Signal
Protein
Synthesis
PCNA Expression
Translation
Initiation
DNA Replication
Lymphokine
Genes
Retinoblastoma
Hyperphosphorylation
Cyclin A
Expression

Figure 2.8: Intracellular pathways of sirolimus action downstream of sirolimus-FKBP complexing with the mammalian target of rapamycin (mTOR) (modified from Dr Suren Sehgal with kind permission)

The sirolimus-FKBP complex does not inhibit calcineurin or cytokine transcription, but interferes with the translation of mRNAs encoding for cell-cycle regulators after binding to mTOR. Figure 2.8 demonstrates the complex downstream pathways that the sirolimus-FKBP-mTOR complex inhibits. In addition to p70S6 kinase, other growth factors for cell cycle progression are inhibited, including the translation inhibitor 4E-BP1(*190*) and the eukaryotic translation initiator protein 4G1 (eIF4G1)(*191*). This control may be either direct or indirect, since TOR also regulates the phosphatase PP2A(*192*).

The development of sirolimus and its accelerated introduction into clinical practice after many years of dormancy in the laboratory, may be one of the most significant contributions to clinical transplantation research in the recent decade. The lack of nephrotoxicity with sirolimus with enhanced immunologic potency represents a major potential advantage over calcineurin inhibitors. The anti-proliferative properties of sirolimus may theoretically confer protection against chronic rejection in transplant recipients, although this remains to be confirmed(*193*).

Initial data from phase II clinical studies of sirolimus in combination with cyclosporine and glucocorticoids after kidney transplantation were compelling–with rejection rates reported at 7.5%(*194*). However, rejection rates were higher (16-19%) in a larger randomized controlled study where sirolimus was given as a fixed dose and not adjusted according to target trough levels(*195, 196*). Impaired renal function, raised blood pressure and hyperlipidemia were concerns raised by this trial; it appears that the increased susceptibility to nephrotoxicity and hypertension may have been due to an interaction between sirolimus and cyclosporine that intensified the action of cyclosporine(*195, 196*).

With an acceptable toxicity profile and markedly lower rejection rates in the absence of evidence of excessive immunosuppression (viral and fungal sepsis, lymphoproliferative disorder or malignancy), sirolimus currently represents a significant advance to the armamentarium. MacDonald recently reported the results of an international phase III randomized controlled trial of sirolimus + CsA + steroids in 576 renal allograft recipients(*197*). Rates of biopsy-proven graft rejection were highly significant between groups, with a 41.5% rejection rate in the placebo group compared with 24.7% in the 2 mg per day sirolimus group, and 19.2% in the 5mg per day group. There was no increase in incidence of infection or malignancy in the sirolimus treated groups. Results of this study further highlight a need to control sirolimus dosing more accurately through serum trough level monitoring to eliminate potential variability between patients and further lower potential rejection rates(*198, 199*).

In liver transplantation, Watson and colleagues demonstrated low rates of acute rejection when sirolimus was combined with CsA, but when patients were converted to sirolimus monotherapy by three months post transplant rejection rates increased, suggesting that sirolimus monotherapy might be inadequate for most patients at least in the first year post transplant(*200*). Furthermore, Trotter *et al* found that a three-day rapid steroid taper together with sirolimus and Neoral™ or tacrolimus maintenance led to 30% rates of acute rejection compared with 70% in historic controls(*201*). Two recent further studies have shown that calcineurin inhibitors may be withdrawn successfully when sirolimus or combined sirolimus and mycophenolate are given(*152, 202*). Hong *et al* further showed that induction treatment with an anti-CD25 (anti-IL2R mAb) led to improvement in early renal dysfunction after renal transplantation, allowing delayed introduction of cyclosporine to facilitate recovery of ischemia/reperfusion related renal injuries(*203*).

Concerns were initially expressed that sirolimus and tacrolimus could not be given in combination, related to their shared molecular morphometry and competitive binding to FKBP12 and FKBP25 cellular entry binding proteins, with evidence of *in vitro* interference(*194*). Vu *et al* clearly demonstrated however that these concerns were not realized *in vivo*, and rat heart allografts demonstrated synergistic potentiation of the action of both drugs when used in combination(*204*). They further hypothesized that clinical relevant doses of either drug fails to saturate an abundance of FKBP12 potential binding capacity of these proteins. This was subsequently confirmed in a primate renal allograft transplant model by Qi *et al*, where synergistic prolongation of renal allograft function was observed in the combined low-dose tacrolimus + sirolimus treated group(*204, 205*). McAlister *et al* used the combination of low-dose tacrolimus with sirolimus and glucocorticoids in an initial series of 32 liver, kidney and pancreas transplant recipients, and found evidence of acute rejection in less than 5% of cases – an unprecedented low rate of rejection in any previous clinical transplant experience(*206*). Their pancreas transplant recipients also received induction therapy with anti-thymocyte globulin(*207*). The sirolimus + low-dose tacrolimus + three-month steroid therapy combination has since been used in over 110 liver, kidney and pancreas recipients at the University of Dalhousie, NS Canada, where the treatment was well-tolerated, and rates of acute rejection were as low as 3%(*208*).

As significant improvements have occurred in the short term outcomes of transplant recipients, issues surrounding long-term morbidity are rapidly becoming major issues in transplantation. Dyslipidemia related to sirolimus therapy has generated concerns, with the potential for increased cardiovascular morbidity and potential for "*death with a functioning graft.*" While the long term risks of sirolimus-related dyslipidemia are unknown at present, emerging preclinical and early clinical data suggest that the atherogenic impact of sirolimus-dyslipidemia may be offset by the potent inhibitory actions of this drug in preventing atherosclerosis. Preliminary evidence for this paradigm shift comes from two sources:

a) Adelman *et al* recently reported a dramatic reduction in atherosclerosis in Apo E-deficient mice despite a 30% elevation in serum LDL-cholesterol(*209*). This change was associated with an increase in IL-10, suggesting that the protective effects on the aortic wall were related to a Th1/Th2 shift to an anti-inflammatory state(*209*), and

b) Patients that received coronary stents locally coated in sirolimus for advanced atherosclerosis maintained long term patency, whereas 30% of non-coated stents occluded within the first three months of implantation(*210*). Both of these observations support the concept that sirolimus may be protective against atherosclerosis both in the transplant and general population. Clearly more detailed, controlled prospective clinical studies are merited to further explore this key issue.

2.10.1 EFFECTS OF SIROLIMUS ON ISLET FUNCTION

Fabian *et al.* studied the *in vitro* and *in vivo* effects of sirolimus monotherapy on mouse islets(*211*). They found no impact of sirolimus on glucose stimulated insulin secretion after 24 hours of culture (10-100 ng/ml), but there was significant deterioration after 72 hours of culture in sirolimus at high concentration (100 ng/ml). Mice treated with 0.1-0.3 mg/kg/day i.p. for 7 days had significant prolongation of islet allograft survival. Adverse impact on glucose homeostasis was demonstrated at 10 to 50 times the effective anti-rejection dose, without evidence of end-organ damage.

Whiting et al. found that rats treated with sirolimus for 14 days at high dose (1.5 mg/kg/day i.p.) developed hyperglycemia, and that this effect was exacerbated by addition of CsA(*212*).

Yakimets *et al.* demonstrated prolongation of canine islet allograft survival when sirolimus (0.05 mg/kg/day i.m.) was given in combination with sub-therapeutic CsA (target range 250-350 µg/L HPLC), whereas either drug given alone did not benefit survival, proving strong synergism of these agents in combination(*213*). A marked correlation was seen between blood levels of sirolimus and graft survival, with blood levels > 10 µg/L (HPLC) proving to be beneficial(*199, 214, 215*). No significant hepatic or renal toxicity was noted, and there was no evidence of deranged glucose homeostasis. Shibita *et al* further demonstrated that temporary sirolimus and CsA treatment combined with deferoxamine prevented islet allograft rejection in adult pigs(*216*).

Kneteman *et al.* have addressed the metabolic impact of sirolimus and CsA after chronic administration in dogs bearing intrasplenic islet autografts (*79*). Frequently sampled glucose tolerance tests performed at one month of sirolimus monotherapy revealed significant *improvement* in KG, due in part to increased basal and stimulated insulin secretion. Insulin clearance was also reduced by 13%, prolonging insulin half-life while on sirolimus – changes that were reversible on discontinuation of therapy. When sirolimus was given in combination with CsA, the direct metabolic benefit of sirolimus was less, but the elevated total insulin response to glucose persisted. There was no evidence of adverse impact, and these findings clearly have promising implications for the role of sirolimus combined with calcineurin inhibition for clinical islet transplantation (*79*).

The future of clinical islet transplantation will be critically dependent on successful protocols for tolerance or near-tolerance induction, which will be an essential pre-requisite to move islet-alone transplantation earlier in the course of the disease. It is predicted that sirolimus will play a major role in future protocols that will transition towards a near-tolerant state. Recent evidence demonstrates that the tolerance induced via costimulatory blockade may be critically dependent upon IL-2 mediated apoptosis of activated T-cells, and calcineurin-inhibitor therapy may interfere with these mechanisms to prevent induction of stable tolerance(*217-219*). Li and colleagues demonstrated that conventional immunosuppression with calcineurin inhibitors effectively prevented stable tolerance induced by costimulatory blockade(*218*). It was further shown that sirolimus did not block the active tolerance process, but treatment with sirolimus plus co-stimulation blockade resulted in massive apoptosis of alloreactive T cells and produced stable skin allograft tolerance, a very stringent test of allograft tolerance(*218, 219*). Interestingly, tolerance achieved through mixed chimerism may be less susceptible to interference from calcineurin-inhibitors, and two patients receiving combined bone marrow + delayed same-living donor kidney transplants have achieved stable tolerance despite temporary cyclosporine therapy(*49*). Aagard-Tillery *et al* showed that sirolimus can directly inhibit CD40 pathways, further suggesting that this drug may help to induce a near-tolerant state when used with appropriate induction protocols(*220*). The development of calcineurin-inhibitor free immunosuppression may prove to be beneficial in future tolerance projects, and therapies involving sirolimus may be key to the early success of this approach.

2.11 ANTI-INTERLEUKIN-2 RECEPTOR ALPHA BLOCKADE

Extensive trials have demonstrated efficacy of monoclonal antibodies directed against the IL-2 receptor alpha chain for induction therapy in transplantation. In the resting state, only the beta and gamma chains of the IL-2 receptor are expressed on T cells. In the activated state, the alpha chain (CD25) becomes expressed(*221*). Since the IL-2 receptor alpha chain is expressed only by activated lymphocytes, this provides more specific and targeted immunosuppression. Anti-CD25 mAb's bind to the exposed alpha chain, preventing downstream phosphorylation of STAT5(*221*).

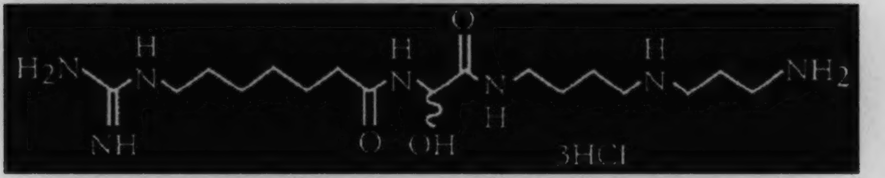
Initial clinical trials used a rodent antibody to IL-2R, and were as effective but better tolerated than anti-thymocyte globulin, permitting lower target levels of cyclosporine to be given(*222, 223*). The development of anti-idiotypic antibodies and short plasma half-life limited treatment courses to only a few days after transplant. Chimeric and humanized versions of the anti-IL2R mAb were subsequently developed, with only the original antibody binding sites being of rodent origin, and the remaining portions are human. Two antibody preparations, basiliximab (chimeric) and daclizumab (humanized), have been evaluated in phase III clinical trials (*224, 225*). In both trials, therapy was compared with placebo together with cyclosporine and glucocorticoids, and azathioprine in the daclizumab trial. In both trials, the antibodies were well tolerated without cytokine release phenomena, and reduced the incidence of acute rejection by approximately 35% without any increase in infectious or malignancy-related complications. These results were maintained at one year post transplant, with 27% acute rejection rates in the daclizumab arm compared with 47% in the placebo control group(*226*). An interim analysis of a large multicenter trial comparing a two-dose daclizumab regimen with no antibody induction in whole pancreas transplantation has demonstrated lower acute rejection rates and improved graft outcome, without associated increase in infectious or malignancy-related complications(*115, 118*).

Tran *et al* reported beneficial impact of daclizumab therapy in a calcineurin inhibitor free regimen together with mycophenolate and glucocorticoids(*227*). Serum creatinine levels were lower in patients where no calcineurin inhibitors were given, and the rates of acute rejection were 31%, and occurred around the tenth post transplant day(*227*).

The risk of post transplant lymphoproliferative disorder (PTLD) is much lower than seen with previous T-cell depletional therapies such as OKT3. Indeed, a cohort of patients were given repeated monthly courses of daclizumab for over a year for recalcitrant psoriasis, and the treatment was efficacious and without detrimental side effects(*228*)

There is no evidence to suggest that anti-IL2R alpha mAb treatment would be damaging to islet function, although this has not been formally tested. The addition of short term or protracted course treatment would likely be highly beneficial for clinical islet transplantation, since they offer the potential a) to spare steroid use, b) to minimize dependence on high dose diabetogenic calcineurin inhibitor therapy, and c) may further preserve islet allograft function by reducing potential risk of acute rejection.

2.12 15-DEOXYSPERGUALIN



15-Deoxyspergualin (gusperimus) (DSG) is a synthetic analogue of a natural protein antibiotic called spergualin produced by the soil bacterium *Bacillus laterosporus*, first isolated from soil samples collected from Ohirasan, Japan(*229*). It was developed initially as an antileukemic drug until Umezawa discovered its immunosuppressive properties in 1985(*230*). DSG is now recognized as a potent anti-inflammatory and immunosuppressive agent with diverse but distinct mechanisms of action, recently reviewed by Kaufman et al(*231*) DSG prolonged survival of heterotopic heart allografts in rats (5 mg/kg/day i.p.)(*232, 233*). Extensive small animal studies have shown benefit of DSG in prolongation of allograft and xenograft survival in heart, liver, kidney and islet models(*234-236*) Initial studies using DSG in kidney allografted dogs (0.6 mg/kg/day s.c.) demonstrated improved graft survival, but led to fatal gastrointestinal side effects(*237*). Monkeys treated with DSG 4-8 mg/kg day i.v. for 10 days followed by 2 mg/kg/day thereafter showed long-term survival of kidney allografts, and was associated with milder side effects than in the dog(*232*). Extensive testing in pre-clinical animal models has shown that DSG is effective both in suppressing and reversing established allograft rejection when used alone or in combination with CsA.

DSG is a pro-drug, and binds to an intra-cytoplasmic immunophilin for activation; its mechanism of action is distinct from CsA and FK-506, however. Nadler *et al.* have shown that DSG binds specifically to Hsc 70, a constitutive member of the heat shock protein 70 family (HSP 70)(*238*). The DSG-Hsc 70 complex may affect folding and unfolding of cytoplasmic protein, and thus interfere with binding and intracellular processing of antigenic peptides(*237, 239*). Most recent data indicates that DSG's predominant immunosuppressive effects are mediated by inhibition of surface expression of Class II MHC on professional antigen presenting cells. Heat shock proteins (notably hsc70) have been implicated in the chaperoning of invariant chain peptides in the endocytic pathway of class II peptide processing(*240-244*).

DSG reversibly inhibits T cell maturation from G0/G1 to S/G2 *in vitro*, and suppresses antigen-driven induction of cytotoxic T cells (CTL) and lymphokine-activated killer (LAK) cells(*245, 246*). DSG specifically inhibits macrophage/monocyte antigen processing and presentation *in vitro*–an effect that is independent of IL-2, class II expression or co-stimulatory response to IL-6 or anti-CD28(*239*). DSG down-regulates class I expression, and inhibits IL-1 and MAF in macrophages *in vitro*(*246*). Furthermore, DSG has been shown to inhibit surface Ig expression on B-lymphocytes, and inhibits B cell differentiation, giving this agent a truly diverse but unique profile of action(*247*).

Thus, DSG suppresses both humoral and cellular immune pathways, and also has specific inhibitory effects on antigen presentation and macrophage function, that may be critically implicated in primary non-function of islet grafts(*231, 234, 248*).

A major drawback for the clinical applicability of DSG is that it is only available currently in parenteral form. While soluble in water, DSG is very poorly absorbed from the gastrointestinal tract. Until this is overcome, the clinical application of DSG will be limited to induction immunosuppression and to in-hospital treatment of acute rejection.

Extensive phase II clinical trials of DSG have been conducted in renal allograft recipients in Japan, and demonstrated that 5 mg/kg/day for 7 days was effective in 77% of cases of acute rejection, and was able to reverse 11 of 13 cases of steroid-resistant rejection(*237, 249*). DSG-related complications included temporary facial paresthesia and leukopenia, which were related both to dose and duration of therapy. Gastrointestinal side effects were minimal.

DSG has shown promise in combined induction therapy in a small series of 7 ABO-incompatible living-related pediatric renal allografts in Japan. Recipient splenectomy, plasmapheresis, DSG, ALG, CsA, steroids and azathioprine led to no incidence of vascular rejection, and 100% patient and graft survival at 44 months(*250*). The role of DSG is difficult to estimate in the context of such an aggressive inductive regime, and the dangers of over-immunosuppression may require a larger series to become evident.

2.12.1 EFFECTS OF 15-DEOXYSPERGUALIN ON ISLET FUNCTION

Strandell and colleagues found that DSG (2.5 mg/kg/day i.p.) reduced the degree of insulitis in mice treated with streptozotocin(*251*); glucose homeostasis was unaffected in control animals. Islets cultured in 4 mg/l DSG, equivalent to the anti-rejection dose *in vivo*, did not impair glucose-stimulated insulin secretion. Higher DSG exposure *in vitro* for 7 days (5-10 mg/l) induced a dose-dependent functional impairment, with morphological evidence of islet degeneration. Xenos *et al* demonstrated that physiological concentrations of DSG did not impair insulin release in isolated rat or human islets, and studies of sub-therapeutic islet mass in rodents significantly increased rates of insulin independence from 22% to 75% with the addition of DSG(*252*). Treatment of normal rats with up to 10 mg/kg/day for 7 days did not affect insulin secretion or glucose disposal. After more chronic exposure, and despite evidence of systemic toxicity from DSG at a dose of 5 mg/kg/day in the rat (weight loss, respiratory failure, sepsis and liver impairment), Jindal *et al* found that glucose tolerance tests and pancreatic insulin content were unaffected(*253*).

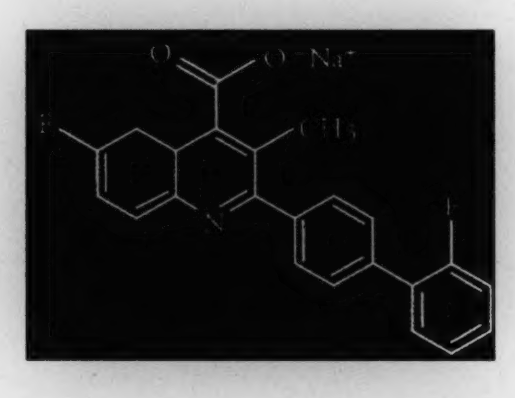
Spurred on by a lack of detrimental effect on islet function, islet allo- and xeno-transplantation have been major test beds for proving efficacy of DSG. Kaufman *et al* found that low dose DSG (0.6 mg/kg/day i.p.) improved initial function of islet isografts in mice after transplantation of a marginal islet mass (150 islets), and suggested that this was due to suppression of macrophage-mediated inflammatory mediators by DSG in the early post period(*234, 248*). An additional factor might be a reduction in nitric oxide production early after transplantation(*254*). Kaufman *et al* further showed that survival of mouse islet allografts was significantly improved by DSG (77% vs. 22% controls).

Menger *et al* studied microvascular changes during engraftment of rat islet xenografts into hamster dorsal skin-fold chambers; DSG monotherapy (2.5 mg/kg/day) was unable to abrogate microvascular rejection phenomena(*255*). Nakajima *et al* found that DSG (5 mg/kg/day) prolonged xenograft survival of hamster islets into rat up to 19 days; a dose of 30 mg/kg/day CsA was required to obtain a similar response(*256*). DSG, used either alone or in combination with CsA or FK-506, has been shown by several other investigators to prolong discordant islet xenograft survival, possibly through DSG's effect on antibody production by B cells(*257-260*).

Addition of low dose DSG (0.5 mg/kg/day) to induction ALG, CsA and azathioprine in dog allografts extended graft survival from 10 to over 30 days(*236*). Encouraged by these results, Gores *et al* were able to achieve insulin-independence in two of six diabetic recipients of single-donor intraportal islet allografts, using induction therapy with DSG(*261*). DSG (4 mg/kg/day for 10 days) was combined with Minnesota-ALG (M-ALG) and prednisone. Introduction of CsA and azathioprine was delayed to promote islet engraftment. Non-purified islets were used for these clinical studies, which may have further contributed to an augmented islet engraftment mass after single-donor transplantation.

Groth *et al* performed xenografts of fetal pig islets in 10 diabetic patients, using conventional immunosuppression and a short course of deoxyspergualin(*262, 263*). Patients had evidence of porcine C-peptide excretion in urine for up to 400 days, and one had evidence of morphologically intact islet clusters on renal graft biopsy(*264*). There was no suppression of xeno-antibody response, however(*265*)

2.13 BREQUINAR SODIUM



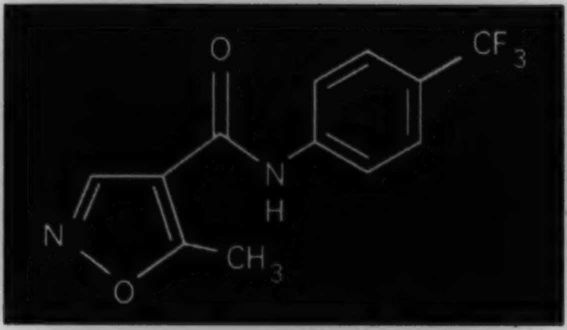
Brequinar sodium (BQR) is a synthetic fluoro-isoquinoline antimetabolite that non-competitively inhibits dihydroorotate dehydrogenase (DHO-DH), a key enzyme in the *de novo* synthesis of pyrimidine. By depriving cells of uridine and cytidine, RNA and DNA synthesis are impaired. In a similar manner to MMF, BQR blocks proliferation of lymphocytes that depend on the *de novo* nucleotide pathway. The anti-proliferative effects of brequinar are less lymphocyte-specific however, and explain a greater incidence of side effects. BQR was initially developed as an antineoplastic drug, but was found to have limited efficacy.

BQR has proven to be effective in preventing rejection of heart, kidney and liver allografts in rats. Cramer *et al.* found that 30 days of treatment with BQR (12 mg/kg) resulted in permanent tolerance of kidney and liver allografts in 50-90% of cases(*266*). Combination of BQR and CsA showed evidence of synergism. Shirwan *et al.* demonstrated that a 3-day course of BQR (12 mg/kg/day) down-regulated a wide variety of local cytokines within rejecting liver allografts, with a shift towards TH_2 profiles in surviving grafts(*267*). The ability of BQR to suppress IgM and IgG synthesis for periods of up to 3 weeks led to investigations that demonstrated efficacy of BQR in sensitized recipient and in xenotransplantation(*268, 269*). These effects may be mediated also in part by BQR-induced inhibition of glycosylation of adhesion molecules(*124*).

Phase I clinical trials in cancer patients revealed that i.v. BQR (15-2,000 mg/m^2) suppressed lymphocyte DHO-DH levels within 15 minutes, and the effect persisted for up to one week. Plasma uridine levels decreased within 6 hours, and rebounded after 4 days. The incidence of toxicity correlated with the level of uridine suppression. Side effects included myelosuppression, mucositis, nausea, vomiting, diarrhea and a painful maculopapular desquamative dermatitis(*270, 271*).

Kahan *et al.* conducted phase I clinical trials of BQR in combination with CsA and steroid in stable renal transplant recipients(*194*). With 24-hour trough BQR levels of < 2 μg/ml, there was < 10% incidence of thrombocytopenia. Experimental studies in mice further suggested that BQR had marked synergism when used in combination with CsA and sirolimus, as shown by median effect analysis. A higher incidence of thrombocytopenia in more extended clinical studies means that this agent is unlikely to be developed further in islet transplantation at the present time.

2.14 LEFLUNOMIDE



Leflunomide (LFN) is a synthetic isoxazole derivative that is transformed *in vivo* to its immunologically active metabolite, A77 1726, which is a selective inhibitor of de novo pyrimidine synthesis. It was developed originally as an immunosuppressive/anti-inflammatory agent in the treatment of rheumatoid arthritis, and only later was it recognized as a potential therapeutic agent for use in transplantation(*272-274*). Initial studies of LFN in healthy volunteers and in patients suffering from rheumatoid arthritis were encouraging, indicating a low toxicity profile. Recent studies in over 2000 patients have confirmed the safety of LFN when administered for over 1 year(*273, 275, 276*). Clinical trials of LFN in transplantation have been set back as the drug was limited for use in rheumatoid arthritis for licensing purposes(*277*).

LFN has emerged as an effective immunosuppressant for experimental allograft and xenograft transplantation. Several studies have confirmed prolongation of skin, kidney, small bowel and heart allografts in rats given LFN (5-30mg/kg), with evidence of synergism if used in combination with CsA(*278-280*). Combination with anti-lymphocyte serum appears to be beneficial in rodent allograft studies(*281*).

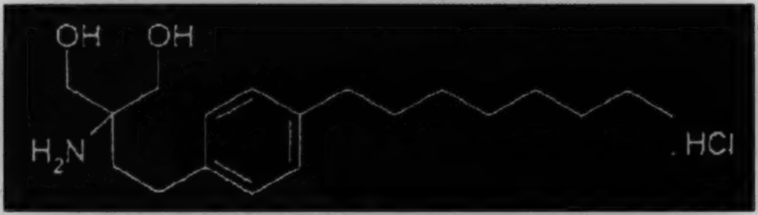
Chong *et al.* found that LFN inhibited T cell proliferation *in vitro* by impairing responsiveness to IL-2 at a post-receptor level, late in the cell cycle (before entry into S phase)(*282*). Lang *et al.* showed that cytokine secretion by T cells was not inhibited by LFN, preserving T cell responses that did not involve clonal expansion(*283*). This specificity may explain the low incidence of infections in pre-clinical studies of LFN.

McChesney *et al.* evaluated LFN in renal transplantation in dogs, and found that increasing oral doses of LFN monotherapy (2-8 mg/kg/day) prolonged graft function progressively to 28 days(*284*). Doses of 16 mg/kg/day led to fatal toxicity in the dog. The combination of LFN (4 mg/kg/day) with CsA (10 mg/kg/day), in doses shown to be ineffective when used alone, was particularly effective, extending mean graft function to 68 days.

One novel property of LFN that distinguishes it from all other immunosuppressants is its inhibitory effect on tyrosine phosphorylation pathways. Xu *et al.* found that LFN strongly inhibited the activity of both $p56^{lck}$ and $p59^{fyn}$ *in vitro*, two key lymphocyte specific tyrosine kinases which mediate signal transduction in T cells after TCR-MHC-peptide interaction or after activation of the IL-2 receptor(*285*). This property may partly be responsible for the anti-tumor effects of this agent(*286*). The precise binding proteins for LFN metabolites have not been identified.

LFN has been used in combination with CsA to enhance fish islet xenograft survival in mice(*287*). Further careful evaluation of LFN in islet transplantation is needed.

2.15 FTY720



FTY720 is a synthetic analogue of myriocin, a metabolite of the ascomycete *Isaria sinclarii*. The agent has a unique mechanism of action, interfering with lymphocyte responsiveness to chemokines, preventing lymphocyte recirculation to the periphery, thereby effectively preventing allograft rejection in rodent islet, skin, heart, liver and small bowel transplantation(*288-293*), and more recently in primate renal transplantation(*294*). An important feature of FTY720 is that it can prevent allograft rejection without inducing generalized immunosuppression; the agent does not inhibit T cell activation or proliferation, cytokine production or B-cell antibody secretion, and does not impair anti-viral memory responses in small animal models(*295*). To date over 100 subjects (stable renal allograft recipients and healthy volunteers) have received single or multiple doses of FTY720. Phase II studies are currently underway in *de novo* renal allograft recipients where 112 patients have been enrolled at 25 sites in Canada, USA, Europe and Brazil (study FTYB201 – personal communication L.Chodoff, Novartis). A randomized, double blind, placebo-controlled trial (FTYB101) has been completed in 20 stable renal transplant patients receiving single oral doses (0.25mg – 3.5mg) for pharmacokinetic analysis. Single doses were well tolerated without significant toxicity. The degree and duration of lymphopenia was dose-dependent, with all lymphocyte subsets similarly affected. Monocyte and granulocyte counts were unaffected. The only possible side effect included a cluster of mild bradycardias in FTY720-treated subjects that may have been independent of therapy(*296*).

It is unclear how this agent will be applied in the clinic, but preliminary data suggests that maintenance rather than an induction approach to therapy may be required to prevent rejection. It is likely that initial studies will be limited to combination therapies with RAD.

2.16 NEW AND EMERGING ANTIBODY THERAPIES WITH POTENTIAL FOR TOLERANCE INDUCTION

The possibility of achieving a permanent state of unresponsiveness (tolerance) to an allograft without the need for chronic immunosuppression remains an important focus in transplantation research. However attainment of a tolerant state is not the only presiding factor limiting the rapid, broader application of islet transplantation in the earliest stages of diabetes, including children. If the risk of chronic long-term immunosuppression could be substantially reduced by a dramatic reduction in degree of systemic immunosuppression, this would accelerate progress towards the ultimate goal. Islet transplantation may prove to be a challenging model to establish tolerance because the dual forces of autoimmune and alloimmune reactivity must both be neutralized, and different mechanistic approaches may ultimately be required to achieve this.

Avoidance of OKT3, anti-thymocyte globulins and other pan-T cell depletional therapies may be important for the future of islet transplantation, both to protect islet grafts from primed destruction in the face of a substantial cytokine storm, but also to minimize the risk of PTLD. The availability of the anti-IL2R alpha mAb's, as discussed above, has been one positive step in this direction. Of the huge number of novel mAb rapidly entering pilot clinical trials, a few promising approaches are highlighted below.

2.16.1 SOLUBLE COMPLEMENT RECEPTOR-1 ANTAGONIST (TP10)

The demonstration by Bennet *et al* of an instant blood-mediated immune response to transplanted islets suggests that potent inhibitors of complement activation might prevent immediate failure of islet engraftment(*297, 298*). A soluble glycoprotein of the human complement receptor type 1 (sCR-1, TP10) has been developed to inhibit both the classical and alternative pathways of complement system activation(*299*). sCR-1 is a single-chain polypeptide produced by recombinant DNA technology that binds to both C3b and C4b, promoting the irreversible dissociation of the catalytic subunits from each of the convertases in both pathways. Therefore sCR-1 is able to inhibit production of activated complement pro-inflammatory (C3a, C5a and C3b) and cytotoxic (C5b-9) protein products of complement activation. Efficacy in transplantation was explored initially to improve function in renal xenografts (hDAF pig to cynomologus monkey). This agent has proven to be beneficial in preserving islet morphology in pig to primate islet xenotransplants(*300*), and in reducing complement deposition. Human Phase I trials are currently underway to determine safety and tolerability of single or multiple dose regimens in inflammatory disease states. Preliminary studies in acute respiratory distress syndrome and acute lung injury suggest that sCR-1 was safe, that the half-life was 70 hours, and significantly inhibited C3 and C5 when given at 3 and 10mg/kg in these patients(*301*). This agent offers the potential to prevent complement-mediated early islet loss in clinical islet transplantation.

2.16.2 ANTI-CD11A (ANTI-LFA-1)

Anti-CD11a is a novel humanized IgG1 mAb directed against the alpha chain of LFA-1(*302*), and is currently entering Phase I clinical trials in renal transplantation and two double-blind, placebo controlled randomized multicenter Phase III trials of efficacy are underway in patients with severe psoriasis(*303*). This antibody prevents the interaction of lymphocyte function-associated antigen (LFA-1) on the surface of T-cells with its ligand, intercellular adhesion molecule (ICAM-1 principally, but also ICAM-2 and ICAM-3) found on the surface of antigen presenting cells(*304*). Previous clinical studies with a murine anti-human anti-CD11a mAb improved survival after bone marrow transplantation(*302*).

LFA-1/ICAM interactions are critical for adherence of leukocytes to endothelial cells, fibroblasts and epithelial cells, and thereby facilitate acute inflammation events(*305*). Blockade of LFA-1/ICAM interactions with humanized anti-CD11a may have particular beneficial impact as an inductive agent in clinical islet transplantation by promoting islet engraftment and preventing inflammatory-mediated cell loss. An additional advantage of anti-CD11a is that it has immunosuppressive properties, interfering with T-cell activation by reducing avidity of T-B cell interactions. In mice, anti-CD11a antibodies have induced tolerance to challenge antigens, reduced severity of autoimmune allergic encephalomyelitis. Gill *et al* demonstrated prolongation of murine islet allograft survival beyond 100 days in 85% of cases (compared with 0% in controls) after treatment with anti-LFA-1(*306*), but found that similar treatment failed to prevent recurrence of autoimmune diabetes in NOD mice.

It remains to be seen whether this promising antibody will have a role in clinical islet transplantation.

2.16.3 CAMPATH-1H

CD52 ANTIGEN

Carbohydrate
Human IgG1 construct
Murine CDR's

Campath-1H is a humanized mAb directed to CD52 determinants on the surface of T-cells, of which there are an estimated 400,000 binding sites per cell. CD52 determinants are present on the surface of all T-cells, and are situated in close proximity to CD45 exons. Waldmann found that optimal T-cell depletion was attained with the anti-IgG1 isoform (1H)(*307*). While the precise mechanisms of action are incompletely understood, it is apparent that this antibody prevents T cell activation indirectly via CD45 signaling events, and does not interfere with T cell receptor activation – which might be highly relevant for future tolerance induction protocols. This treatment has proved to be highly effective in bone-marrow transplantation for T-cell purging to eliminate graft-versus-host disease in over 2000 patients treated in Europe in the treatment of B-cell lymphomas(*308, 309*) Efficacy has recently been demonstrated in non-myeloablative conditioning for stem cell therapy(*310*). Conditioning regimens based on Campath, cyclosporine and donor marrow stem cell therapy provided durable engraftment in 62 of 64 recipients, with macrochimerism demonstrable in 31 cases(*311*). This antibody has been particularly effective in control of autoimmune diseases, including acute vasculitides(*312*), multiple sclerosis(*313*) and in autoimmune cytopenias(*314, 315*). Calne et al used Campath-1H for induction prophylaxis in 31 patients undergoing renal transplantation(*316, 317*). Campath-1H was administered at a dose of 20mg on day 0 and repeated on day 1, and after a 48-hour delay half-dose cyclosporine was initiated without glucocorticoids and without any other maintenance drug therapy. Remarkably, with a mean two-year follow-up, 28 patients have functioning grafts, with an incidence of acute rejection of 12.9%, and with a rate of infectious complications that did not differ from a control series of standard therapy(*316, 317*). The perceived advantages of this approach are steroid-avoidance, maintenance immunosuppression with reduced side effects in a simple cost-saving regimen that is generally well tolerated. The potential role for this agent in islet transplantation is substantial, as effective control of rejection and autoimmunity could be provided in the complete absence of diabetogenic immunosuppression – since both glucocorticoids and calcineurin inhibitors would be eliminated from the regimen.

Kirk *et al* treated 7 living-related kidney transplant recipients with Campath-1H monotherapy initially, giving pre-treatment on day –3, -1 and on day 2 post transplant. They found that Campath was well tolerated and was safe, and led to profound T and B cell peripheral depletion(*318*). In the absence of maintenance immunosuppression, acute rejection was seen, associated with a rise in peripheral monocyte count without change in lymphocyte count. The monocytic infiltrate was associated with augmented

TNF-alpha expression. The subsequent introduction of low dose maintenance sirolimus provided excellent renal function in all cases.

Knechtle et al treated 5 renal transplant recipients (3 cadaveric, 2 living-related) with Campath-1H 20mg on Day 0 and Day 1 together with a single pulse of solumedrol (500mg), and began sirolimus monotherapy from day 1 (level controlled to 8-12ng/ml). All patients have stable renal graft function without any episodes of rejection, and there has been no significant morbidity in over six months of follow-up. This extremely encouraging data suggests that the Campath/sirolimus combination achieves a goal of minimal long-term immunosuppression in kidney transplantation, and is an attractive approach to explore further in islet transplantation.

Extensive clinical experience has shown that first-dose administration of Campath-1H can be associated with cytokine release phenomena, including fever that resolves within 24 hours, mild hypotension, and occasionally urticarial-type rashes. These complications could be minimized by pre-treatment with anti-inflammatory agents including anti-TNF alpha treatment. Campath therapy has been associated with acute rise in TNF-alpha and IFN-gamma, and Campath-mediated perturbations in TNF-alpha may be avoided by anti-TNF-alpha therapy(*313*). The addition of an anti-TNF alpha therapy could therefore potentially enhance islet survival by: a) controlling the TNF-alpha response, thereby reducing the severity of cytokine release symptoms(*313*), promote islet engraftment(*319*), and potentially reduce the risk of monocyte-mediated graft rejection.

2.16.4 NON-MITOGEN CD3 MAB (HOKT3-ALA ALA)

Bluestone and colleagues developed a humanized FcR non-binding anti-CD3 mAb (hOKT3γ_1-Ala-Ala) that targets only activated T-cells, and lacks toxicity previously associated with conventional anti-CD3 mAb therapy. The mAb was genetically engineered from murine OKT3 mAb by grafting the six complementarity-determining regions into a human IgG1 mAb, and where the C_H2 region was altered by site-directed mutagenesis to avoid T-cell activation(*320, 321*). These changes to the OKT3 molecule have been designated as "ala-ala", in reference to the carbohydrate changes made to the Fc portion of the antibody. FcR non-binding anti-CD3 Abs have short-lived effects on naïve T-cells but deliver a partial signal in activated T-cells resulting in clonal inactivation of Th1 cells while sparing the suppressive Th2 T cell subset. This approach was developed to selectively inhibit and tolerize the inflammatory subset of auto- and allo-reactive Th1 cells, thus preventing inflammation and restoring peripheral self-tolerance. Short-term immunotherapy of overtly diabetic NOD mice with an anti-CD3 mAb restored durable self-tolerance to autoantigens and prevented autoimmune recurrence in syngeneic islet grafts – this ability to restore self-tolerance in the presence of an ongoing autoimmune response is unprecedented(*322, 323*). A 5-consecutive-day treatment induced a complete and durable remission beyond 8 months in follow-up, and subsequent syngeneic islet grafts were not rejected. Preliminary data from a type 1 diabetes primary prevention trial in patients treated with hOKT3γ_1-Ala-Ala within six weeks of diagnosis, suggest that marked improvement in Phase 1 insulin release can be restored (K. Herold, personal communication). Twenty one patients have received therapy in this trial to date, with 12 patients followed beyond one year. 8/12 patients demonstrated more C-peptide production than was evident at time zero, compared to

2/10 in the control group, and this was associated with a 40% reduction in HbA1C at 6 months, compared with pre-treatment controls. Side effects have been mild, including mild self-limiting rash, and an anti-idiotypic antibody response in 1/3rd of cases. Serious adverse events were not observed in Phase I trials in treatment of clinical renal transplant rejection, psoriatic arthritis, and new onset type 1 diabetes. Steroid-resistant renal transplant rejection was promptly reversed in 5 of the 7 patients treated in a Phase I trial performed at the University of Chicago(*324*). A significant increase in serum IL-10 was noted following the initial treatment in these patients. Phase II studies are currently underway in psoriatic arthritis to further evaluate efficacy.

Collectively, these results highlight the hOKT3γ_1Ala-Ala mAb as a new and distinct immunotherapeutic agent that selectively inhibits an inflammatory subset of activated T-cells. In addition, the pre-clinical results demonstrating that the anti-CD3 therapy selectively inhibits primed, IFNg/TNFa producing Th1 cells while sparing the IL-10 T-cells holds great promise for protecting human islet allografts from alloimmune and autoimmune destruction.

Hering has recently reported 3 islet-alone type 1 diabetic recipients with hOKT3γ_1-Ala-Ala together with tacrolimus and sirolimus(*325*). Remarkably, all three patients achieved insulin independence with current follow-up beyond 100 days without rejection after single-donor islet infusions of mean 11,600 IE/kg. These high yields reflected outstanding isolations infused into low-weight recipients. This encouraging preliminary data suggests that therapy is safe, and may be combined with steroid-free immunosuppression.

2.17 SYNTHESIS OF CURRENT STATUS OF IMMUNOSUPPRESSION, AND CONCLUDING REMARKS

The selection of optimal immunosuppression for islet transplantation provides a unique challenge. The combination of agents must be highly potent–the lack of a rejection marker means that the luxury of graft rescue from the ravages of rejection is not an option at present. The chosen regime must have an especially low risk of over-immunosuppression – islet transplantation may afford freedom from injected insulin and may indirectly enhance longevity, but is not a life-saving procedure in itself.

Current mainstream immunosuppression with CsA, azathioprine and steroids is inadequate to match the demands of high potency at low risk. More specific therapies are needed if islet transplantation is to have more widespread application in the diabetic population. The constraints of safety dictate that novel therapies be tested thoroughly in multicentre trials of life-saving organ transplantation before they may be applied freely to islet transplantation.

The additional challenge for islet transplantation is a need to define the profile of adverse impact on islet function any new regime may have–and this demands systematic appraisal both in pre-clinical animal models and at the bedside too. Multicentre co-operation in randomized trials of immunosuppression in islet transplantation would enhance our knowledge at a faster pace. It would ensure too that as new immunosuppressive drugs proliferate, their safety and efficacy in the clinic is assured over the drugs they replace.

Tacrolimus offers greater potency, but at the price of increased toxicity and greater diabetogenic potential, and is unlikely to be an attractive alternative to CsA for islet transplantation. Azathioprine has low diabetogenic potential, but is non-selective and only modestly effective as an immunosuppressant. Glucocorticoids have the greatest adverse impact on islet function. Substitution of azathioprine by MMF offers a 50% reduction in acute rejection risk, but may impair islet graft function. A marked reduction in steroid requirements would likely counteract any adverse impact of MMF. Sirolimus in combination with CsA and low-dose steroids offers modest potency. The combination of sirolimus with low-dose tacrolimus and no steroids is a further possibility for islet transplantation with the added advantage of enhancing islet graft function. However, the safety profile of rapamycin of this combination is not fully known at the present time.

A broader application of deoxyspergualin will await development of an oral formulation, but an extensive experimental base and limited clinical experience suggest that DSG may be used effectively to optimize induction therapy. Mizoribine is similar in action to MMF, but is less selective and is potentially more mutagenic, and is thus unlikely to have application outside of Japan. Brequinar awaits more detailed evaluation in phase I/II trials of kidney and liver transplantation. Leflunomide is currently undergoing extensive phase III trials in rheumatoid arthritis patients, but awaits further evaluation in transplantation.

The role for inductive immunosuppression with polyclonal antibody preparations remains undefined, but is likely to become more restricted with the introduction of newer and more potent drugs. The only monoclonal inductive agent in widespread clinical practice is OKT3. The massive cytokine release with its toxic potential for islets, combined with increased risk of CMV infection and lymphoproliferative disorder, leave OKT3 unfavored as a suitable agent for islet transplantation. The horizons opened up by an explosive increase in highly specific newer monoclonal uncouplers of the allograft response offer great potential, but await more thorough evaluation.

As our understanding of mechanisms to promote transplantation tolerance expand, attention must shift to ensure that the newer immunosuppressive agents do not interfere with induction of allograft-specific anergy.

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CHAPTER 3:

DEFINING OPTIMAL IMMUNOSUPPRESSION FOR ISLET TRANSPLANTATION BASED ON REDUCED DIABETOGENICITY IN CANINE ISLET AUTOGRAFTS – SYNERGISTIC TOXICITY FROM COMBINED GLUCOCORTICOID AND CALCINEURIN INHIBITOR THERAPY.

NOTE: A previous version of this chapter is currently under review in Transplantation, and authors on this paper include: Shapiro AMJ, Hao EG, J, Lakey JRT, Finegood D, Rajotte RV, and Kneteman NM.

3.1 ABSTRACT

The recent results of clinical islet transplantation have improved substantially with the introduction of a more potent but less diabetogenic immunosuppressant protocol. The successful development of this protocol was based in part on the outcomes of studies reported herein, addressing the diabetogenic potential of a series of immunosuppressant agents used alone or in combination in a canine islet autograft model. While it is recognized that failure to achieve long-term insulin independence in human islet allotransplantation has been multi-factorial, with low engraftment mass, acute or chronic rejection, autoimmune recurrence, loss of islet-acinar integrity, heterotopic site, denervation and insulin resistance all being implicated to varying degrees. Avoidance of diabetogenic immunosuppression has been pivotal to the enhanced outcomes of clinical islet transplantation. We herein explore the effects of clinically relevant doses of cyclosporine or tacrolimus when given alone or in combination with glucocorticoids on long-term canine islet autograft function.

Method: Dogs (n=8) underwent total pancreatectomy, islet isolation and intrasplenic autotransplantation, and were normoglycemic with stable long-term graft function three months to eight years post-transplant. The frequently sampled IV glucose tolerance test (FSIGT) was performed pre-drug (baseline), at 1 month of therapy (on drug), and again 1 month after withdrawal of therapy (post). Derived variables of glucose decay, insulin responsiveness, insulin sensitivity and glucose effectiveness were computed using a modified Bergman minimal model kinetics, for low or high dose

prednisone monotherapy, cyclosporine (Neoral^{TM}), tacrolimus or combined with glucocorticoids.

Results: Monotherapy treatments with low or high dose prednisone, Neoral^{TM} or tacrolimus had minimal impact on islet autograft function. The combination of Neoral^{TM}/prednisone led to a marked impairment in glucose decay (25% decline from 1.77 ±0.2 to 1.24 ±0.2, $p<0.05$), without significant change in insulin responsiveness or glucose effectiveness. However insulin sensitivity was markedly impaired while on therapy (7.10 ±1.2 to 3.10 ±0.5, $p<0.01$). Importantly, glucose decay and insulin sensitivity failed to return to baseline after withdrawal of therapy. The combination of tacrolimus and glucocorticoids led to permanent and irreversible diabetes in all recipients ($n=6$, $p <0.001$). Similar treatment of healthy control dogs led to a 44% decrease in glucose decay ($p < 0.01$).

Conclusions: While monotherapy treatment with calcineurin inhibitors or prednisone did not significantly impair islet autograft function, the combination of calcineurin inhibitor with glucocorticoid led to marked impairment of graft function. The combination of tacrolimus and glucocorticoid induced permanent islet autograft failure. Immunosuppression must be specifically tailored for islet transplantation if insulin independence is to be sustained clinically.

3.2 INTRODUCTION

The recent results of clinical islet transplantation have improved substantially with the recent introduction of a more potent but less diabetogenic immunosuppressant protocol, based on sirolimus, low-dose tacrolimus and an anti-CD25 monoclonal antibody in a completely glucocorticoid-free regimen(*1*). The successful development of this protocol was based in large part on the outcomes of the pre-clinical studies reported herein, which specifically address the diabetogenic toxicity of a series of immunosuppressant agents used alone or in combination in canine islet autografts. Failure to achieve long-term insulin independence in patients undergoing clinical islet transplantation for type 1 diabetes is multi-factorial; the combination of a sub-therapeutic islet engraftment mass compounded by detrimental impact of acute or chronic rejection, recurrence of autoimmunity, loss of islet-acinar integrity, heterotopic location, denervation and systemic insulin resistance in the presence of uremia have all been implicated to varying degrees(*2-8*). It has long been recognized that the diabetogenic effects of immunosuppressive drugs may further exacerbate islet graft dysfunction(*9-11*). However relevant impact at physiologically relevant doses has not been clearly defined, and the interactions within combination therapies as applied clinically have not been well characterized to date.

Cyclosporine, azathioprine and glucocorticoids were previously the mainstay therapy for the majority of the 447 previous attempts at clinical islet transplantation, where overall less than 10% of patients maintained insulin independence for longer than one year(*12*). With recent improvements in islet isolation and quantification, the most recent clinical results in islet allograft recipients with type 1 diabetes indicate an islet function rate of 50% (evidence of ongoing C-peptide secretion) and insulin independence rate of 20% with cyclosporine-glucocorticoids based therapy(*13-16*). Cyclosporine has been previously associated with adverse impact on islet revascularization, insulin secretion and insulin sensitivity when given at supra-physiologic dose(*17-20*). Insulin sensitivity may recover after withdrawal of therapy(*21*). Other studies have failed to demonstrate a detrimental impact of cyclosporine monotherapy at clinical dose(*22*). Similar reversible impairment in islet function has been observed for tacrolimus(*23-25*). Tacrolimus has been associated with islet vacuolization and fibrosis in whole pancreas transplantation, with extent correlating with drug level(*26*).

The current study therefore explores the diabetogenic impact of clinically relevant doses of cyclosporine (Neoral™) or tacrolimus (Prograf™) when used alone or in combination with glucocorticoids on the metabolic efficiency of intrasplenic islet autografts in long-term canine recipients. The autograft model provided an opportunity to study the effects of immunosuppressants on islet graft function without being confounded by the effects of autoimmune recurrence or islet rejection. Previous studies by our group had demonstrated no evidence of metabolic dysfunction from physiologic-dose cyclosporine monotherapy in this model, and the addition of sirolimus led to a modest improvement in glucose clearance, increased total and stimulated insulin release in response to glucose with reduced insulin clearance(*27*).

The underlying hypothesis states that the immunosuppressants used to prevent rejection also impair islet graft function, thereby preventing attainment of insulin independence after islet transplantation.

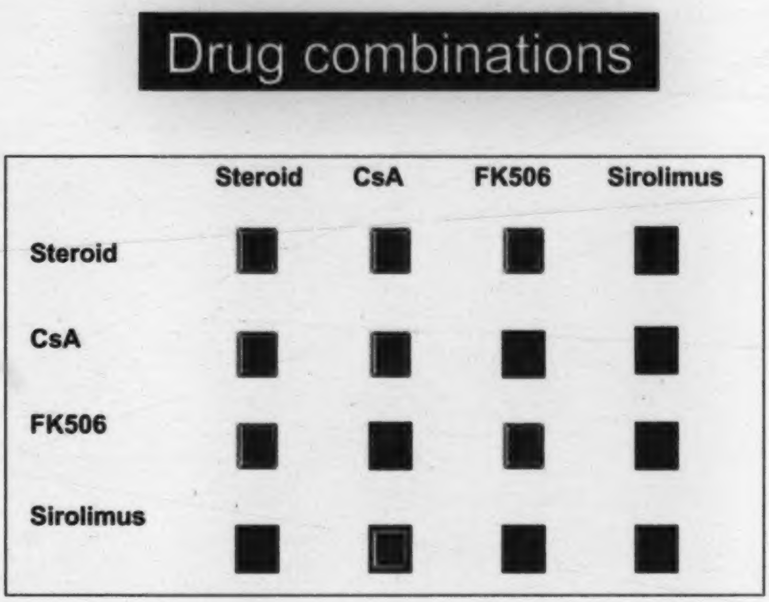
3.3 MATERIALS AND METHODS:

3.3.1 ANIMALS:

Study animals (n=6 per group) consisted of mongrel dogs that had previously undergone successful total pancreatectomy, islet isolation by collagenase digestion, purification on discontinuous Ficoll gradients and reflux of islets into the spleen by retrograde infusion via the splenic vein(*28, 29*). All autograft recipients received an estimated islet implant mass ranging between 3,100 – 6,600 IE/kg (median 5,400 IE/kg), had stable euglycemia with a minimum of three months and as long as eight years (median 3.5 years) post transplant. Animal husbandry followed the strict guidelines of the Canadian Council on Animal Care, with the support of personnel from the Health Sciences Laboratory Animal Services of the University of Alberta.

3.3.2 DRUGS AND ADMINISTRATION:

Glucocorticoid therapy was given in the form of oral prednisone tablets (Apotex, Weston ON, Canada) at low and high dose (0.15mg/kg and 0.4mg/kg per day respectively). Cyclosporine Neoral™ was given in capsule form, as supplied by Novartis Pharmaceuticals Canada Inc., at an initial clinically relevant dose of 5mg/kg/bid orally, with subsequent dose adjustment to maintain target whole blood levels 300-500μg/L appropriate for this canine model(*22, 27*). Parent cyclosporine drug levels were analyzed by fluorescence polarization immunoassay (FPIA, Abbot TDX, Abbot Inc., Abbot Park, IL, USA) and confirmed by HPLC(*30, 31*). Tacrolimus was also given in capsule form (a gift from Fujisawa Canada) at an initial dose of 0.01 mg/kg twice daily, adjusting subsequent dose to achieve therapeutic levels of 10-15µg/L; parent Tacrolimus levels were assayed by a specific monoclonal antibody ELISA (Incstar Inc., Stillwater, MN, USA).



Drug combinations

	Steroid	CsA	FK506	Sirolimus
Steroid	■	■	■	■
CsA	■	■	■	■
FK506	■	■	■	■
Sirolimus	■	▣	■	■

Table 3.1: Summary of drug combinations used to determine impact on canine islet autograft function (red). Note: Cyclosporine monotherapy group and Sirolimus ± cyclosporine combination was evaluated previously by Kneteman *et al* (blue)(*27*). Evaluation of sirolimus + tacrolimus not tested because all grafts failed on preceding combination therapy. .

3.3.3 EXPERIMENTAL PLAN:

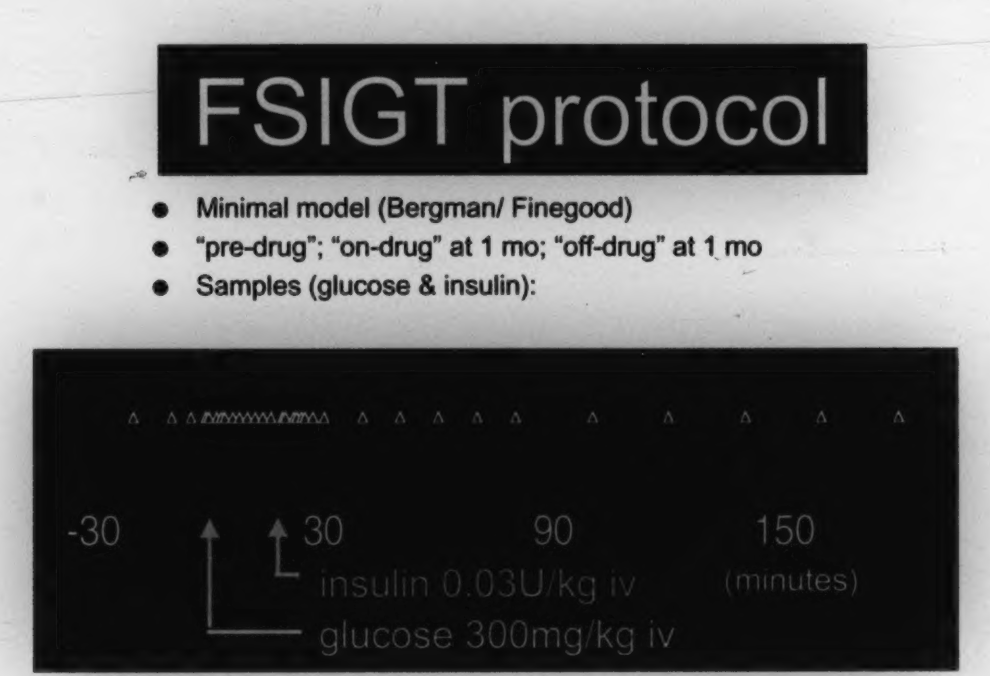
Each of six animals underwent a frequently sampled intravenous glucose tolerance test (FSIGT) at baseline, at the end of one month of drug therapy, and again one month after discontinuation of therapy. The animals were then maintained for a further 1-3 months without drug treatment to confirm baseline graft stability and euglycemia. The animals were randomized to receive initial treatment with low or high dose prednisone, cyclosporine or tacrolimus. Once these studies were completed, animals were further randomized to receive combination therapy with cyclosporine + low-dose prednisone, or tacrolimus + low-dose prednisone. If an animal became overtly diabetic on therapy (fasting glucose ≥ 15 mmol/L on more than two occasions), therapy was withdrawn and maintenance insulin therapy was continued as needed. If diabetes failed to recover within one month of withdrawal of therapy then animals were humanely sacrificed. In this case, replacement islet autograft recipients were included once they had stable baseline graft function and were beyond three months from transplantation.

3.3.4 ANALYSES:

Glucose was measured by the glucose oxidase method using a Beckman Glucose Analyzer II (Beckman Instruments, Brea, CA). Insulin was analyzed in the Muttart Core Radio-immunoassay laboratory by a double-antibody method(*32*). Cyclosporine and tacrolimus were assayed as defined above.

3.3.5 FREQUENTLY SAMPLED INTRAVENOUS GLUCOSE TOLERANCE TEST (FSIGT):

Detailed metabolic studies were performed in stable canine islet autograft recipients using a Bergman Minimal Model method modified by Finegood for application to islet transplantation(*27, 33, 34*). Briefly, awake animals were initially conditioned to a Pavlov frame and subsequently underwent cannulation of a lateral saphenous vein (18-guage catheter) and were left to stabilize for a further 30 to 60 minutes. After drawing basal samples, a glucose bolus of 0.3 g/Kg was infused intravenously and blood samples collected at 2,3,4,6,10,12,14,16 and 19 minutes. At 20 min a bolus injection of 0.03 units/kg of insulin was administered (crystalline zinc Pork Insulin, Connaught, Willowdale ON), and blood for glucose and insulin further collected at 22, 23, 24, 25, 27, 30, 40, 50, 60, 70, 80, 100, 120, 140, 160 and 180 minutes (see figure 3.1)(*27*).

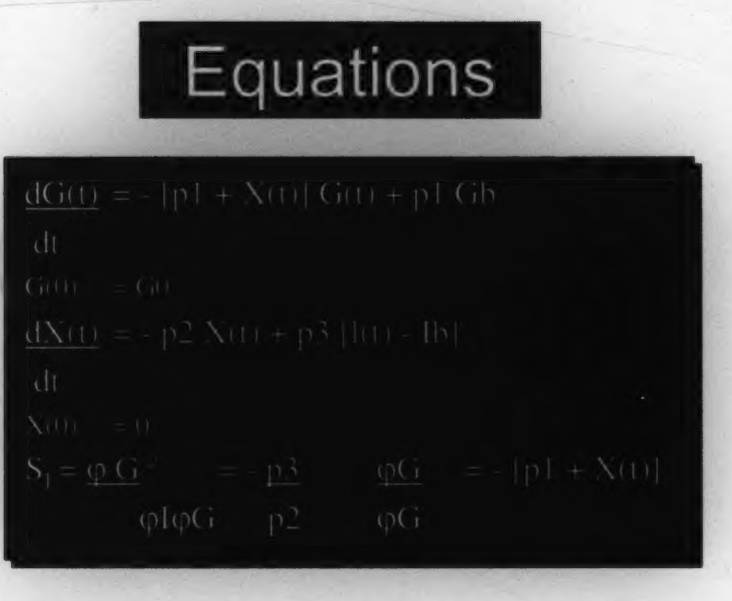


FSIGT protocol

- Minimal model (Bergman/ Finegood)
- “pre-drug”; “on-drug” at 1 mo; “off-drug” at 1 mo
- Samples (glucose & insulin):

Figure 3.1: Summary of FSIGT protocol for glucose and insulin delivery, and sample timing.

MINMOD computer modeling of the detailed FSIGT data allowed calculation of the following variables, calculated according to equations and methods defined by Bergman et al, and subsequently modified by Finegood et al for application to islet transplantation(*33, 34*): Glucose decay constant (K_G), calculated from the regression slope of the log glucose over time; Insulin Response (IR), representing the weighted mean increase in insulin from basal levels, Insulin Sensitivity (SI) defining the effect of insulin in increasing the disposal of glucose in response to hyperglycemia, and Glucose Effectiveness (SG), defined by the ability of glucose to increase its own disposal at basal insulin levels (se table 3.3) (*33, 34*).



Equations

$$\frac{dG(t)}{dt} = -[p1 + X(t)]\,G(t) + p1\,Gb$$

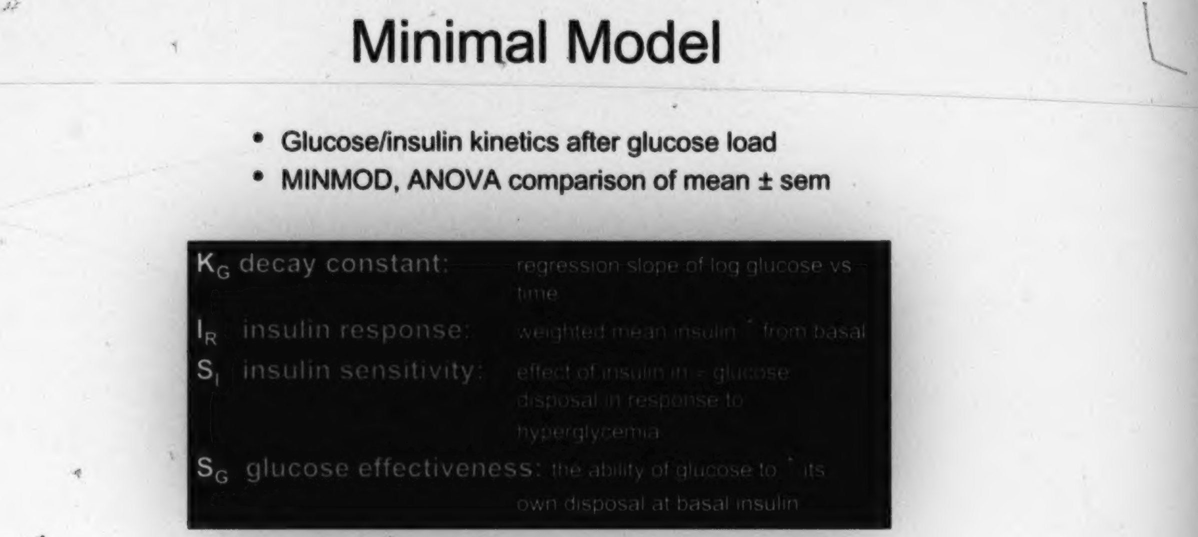
$$G(0) = G0$$

$$\frac{dX(t)}{dt} = -p2\,X(t) + p3\,[I(t) - Ib]$$

$$X(0) = 0$$

$$S_I = \frac{\varphi\,G^2}{\varphi I \varphi G} = -\frac{p3}{p2} \qquad \frac{\varphi G}{\varphi G} = -[p1 + X(t)]$$

Table 3.2: Equations used to calculate secondary variables in the Minimal Model *(after Finegood, Diabetes 1984: 33; 362)*



Minimal Model

- Glucose/insulin kinetics after glucose load
- MINMOD, ANOVA comparison of mean ± sem

K_G	decay constant:	regression slope of log glucose vs time
I_R	insulin response:	weighted mean insulin ˆ from basal
S_I	insulin sensitivity:	effect of insulin in = glucose disposal in response to hyperglycemia
S_G	glucose effectiveness:	the ability of glucose to ˆ its own disposal at basal insulin

Table 3.3: Secondary variables derived from the Minimal Model analysis *(after Bergman and Finegood)*

3.3.6 STATISTICAL ANALYSIS:

Glucose-insulin kinetics from the FSIGT were calculated using the MINMOD computer program, in collaboration with Dr D. Finegood(*34*, *35*). Means were compared using analysis of variance with univariate test hypotheses for within subject paired data for pre-therapy, on drug therapy and one month after discontinuation of therapy. Statistical difference was defined by $p<0.05$.

3.4 RESULTS

3.4.1 CYCLOSPORINE MONOTHERAPY:

One month of therapy with cyclosporine monotherapy did not influence K_G or other parameters of autograft function, with values being statistically similar on therapy versus pre-treatment baseline or after discontinuation, (as shown in figure 3.2 and table 3.4 below). The cyclosporine monotherapy data was derived from a previously published historic control group reported by Kneteman *et al* in the same model*(27)*.

3.4.2 GLUCOCORTICOID THERAPY:

With low dose prednisone (0.1 mg/kg/day) there was no statistical change in K_G or other secondary functional parameter while on therapy (figure 3.3 and table 3.5).

With one month of high dose prednisone monotherapy (0.4 mg/kg/day), surprisingly there was also no evidence of islet autograft deterioration, as measured by K_G or secondary parameters of insulin or glucose kinetics while on therapy compared with baseline or one month after drug withdrawal (figure 3.4 and table 3.5).

However there was a 25% decrease in insulin sensitivity while on high dose prednisone therapy which did not reach statistical significance.

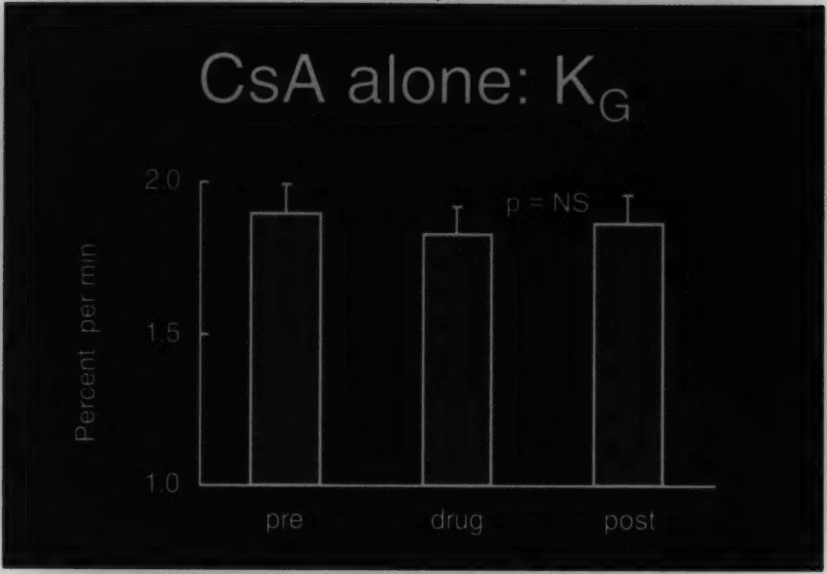
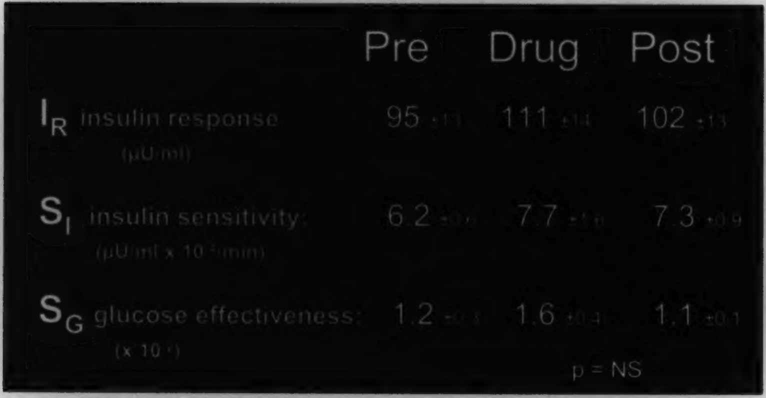


Figure 3.2: Cyclosporine monotherapy does not impair intrasplenic islet auto-transplant function, based on glucose clearance *(K_G,, mean ± SEM) Data for the cyclosporine monotherapy group is from historic controls, previously published by Kneteman et al(27).*

CsA alone



	Pre	Drug	Post
I_R insulin response (μU/ml)	95 ±11	111 ±14	102 ±13
S_I insulin sensitivity: (μU/ml x 10 [illegible]/min)	6.2 ±0.6	7.7 ±1.0	7.3 ±0.9
S_G glucose effectiveness: (x 10 [illegible])	1.2 ±0.3	1.6 ±0.4	1.1 ±0.1

p = NS

Table 3.4: Cyclosporine monotherapy does not secondary variables of graft function after MINMOD analysis *(mean ± SEM, p=NS, ANOVA) (Historic control data, published previously by Kneteman et al) (27))*

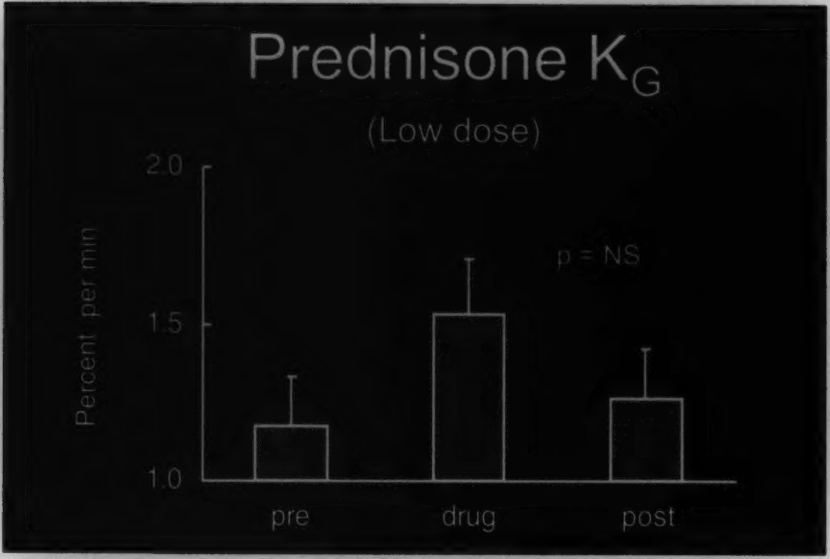
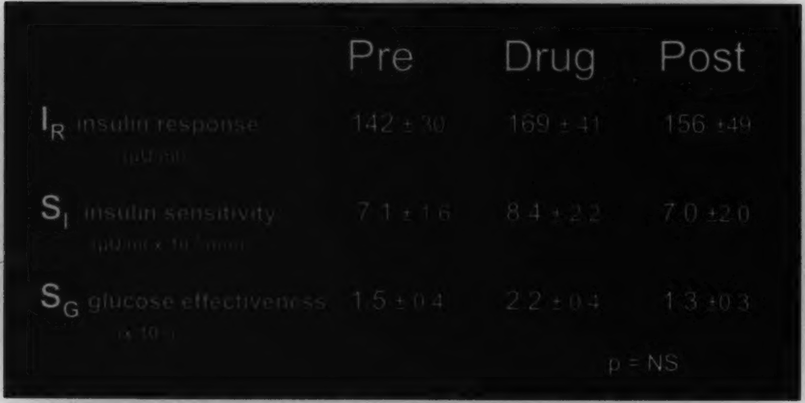


Figure 3.3: Low dose prednisone does not impair intrasplenic islet auto-transplant function, based on glucose clearance (K_G)

Prednisone

(low dose)



	Pre	Drug	Post
I_R insulin response [illegible]	142 ± 30	169 ± 41	156 ±49
S_I insulin sensitivity [illegible]	7.1 ± 1.6	8.4 ± 2.2	7.0 ±2.0
S_G glucose effectiveness [illegible]	1.5 ± 0.4	2.2 ± 0.4	1.3 ±0.3

p = NS

Table 3.5: Low dose prednisone does not impair intrasplenic islet auto-transplant function, based on glucose clearance (K_G)

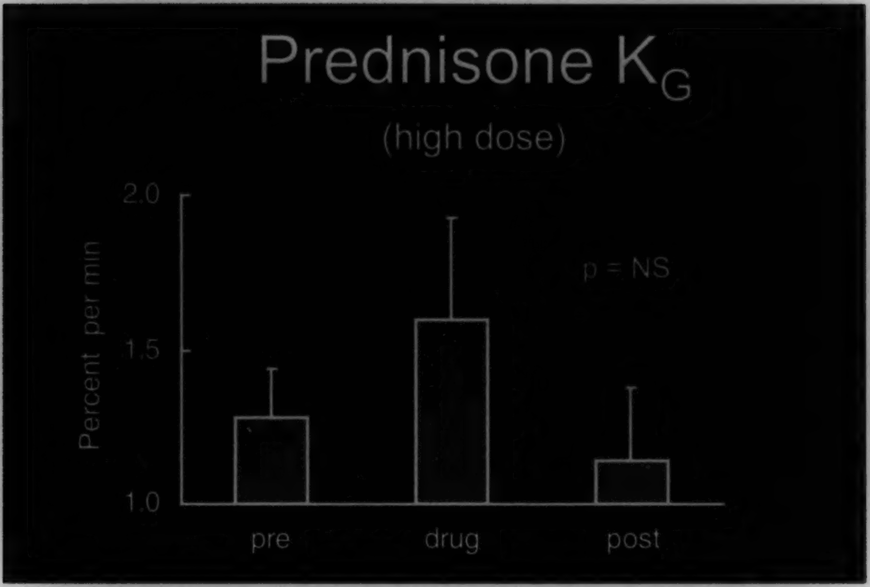
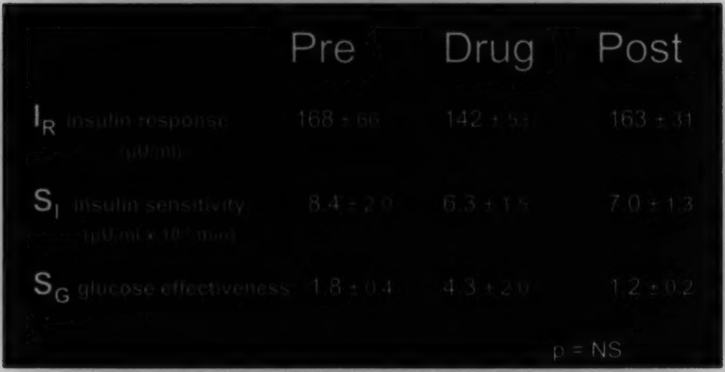


Figure 3.4: High dose prednisone does not impair intrasplenic islet autotransplant function, based on glucose clearance (K_G)

Prednisone
(high dose)



	Pre	Drug	Post
I_R insulin response (μU/ml)	168 ± 66	142 ± 53	163 ± 31
S_I insulin sensitivity (μU/ml × 10^{-4}/min)	8.4 ± 2.0	6.3 ± 1.5	7.0 ± 1.3
S_G glucose effectiveness	1.8 ± 0.4	4.3 ± 2.0	1.2 ± 0.2

p = NS

Table 3.6: High dose prednisone does not impair intrasplenic islet autotransplant function, based on secondary MINMOD parameters.

3.4.3 COMBINATION OF CYCLOSPORINE AND LOW DOSE GLUCOCORTICOID:

While no statistical differences in functional graft parameter were observed with cyclosporine or prednisone monotherapy, the combination of these two drugs caused the K_G to deteriorate by 30.5% ($p < 0.05$, see figure 3.6). Importantly, this defect caused permanent impairment in autograft function, and failed to recover by one month after discontinuation of therapy. This defect was accounted for entirely by a 44% reduction in insulin sensitivity ($p < 0.01$, table 3.7). A comparison of mean glucose decay and insulin response and clearance curves from normal dogs (n=6), islet autograft controls off therapy and in the cyclosporine + prednisone-treated group is shown in figure 3.5 below.

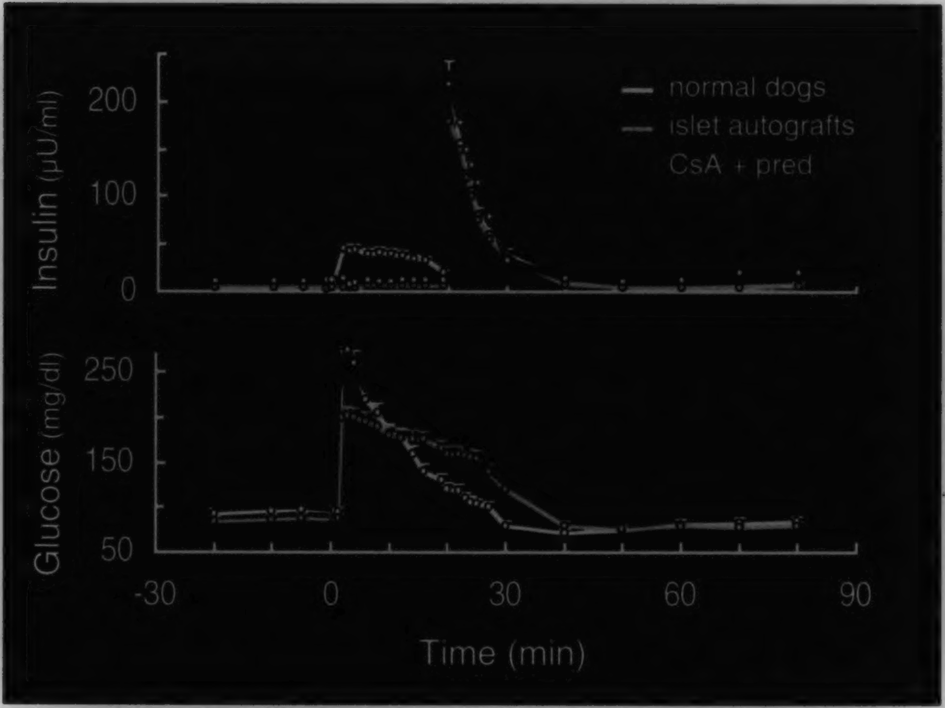


Figure 3.5: Glucose and insulin clearance in the Minimal Model for normal dogs (white), islet autografted recipients (yellow) and islet autografted recipients immunosuppressed with combination cyclosporine/prednisone (red), where there is impaired graft function *(mean ± SEM).*

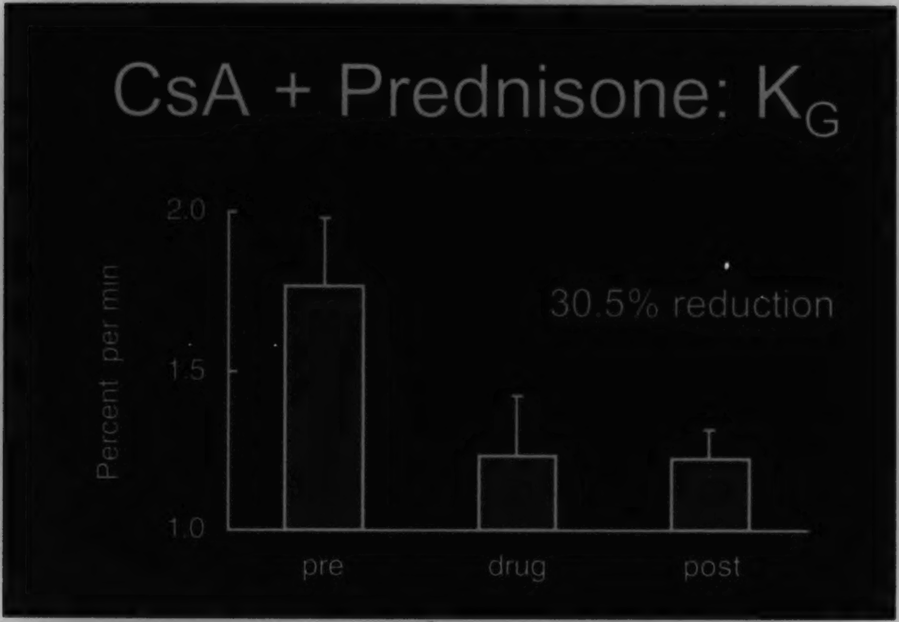
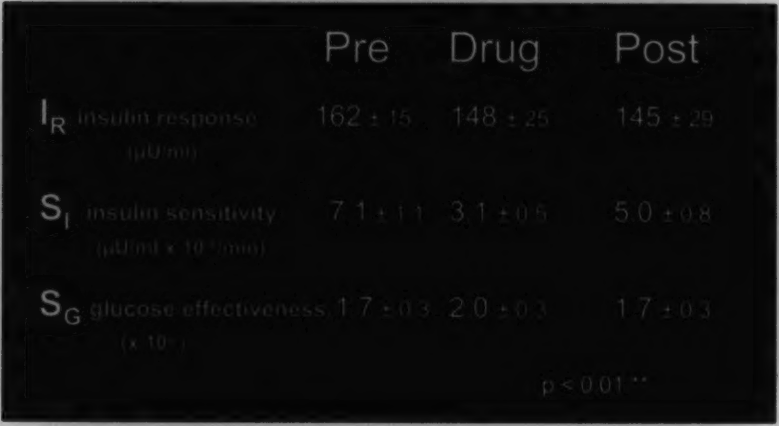


Figure 3.6: The combination of cyclosporine + prednisone led to a significant reduction in glucose clearance (K_G), which failed to recover by one month after discontinuation of therapy.

CsA + Prednisone



	Pre	Drug	Post
I_R insulin response (μU/ml)	162 ± 15	148 ± 25	145 ± 29
S_I insulin sensitivity (μU/ml × 10 [illegible]/min)	7.1 ± 1.1	3.1 ± 0.5	5.0 ± 0.8
S_G glucose effectiveness (× 10 [illegible])	1.7 ± 0.3	2.0 ± 0.3	1.7 ± 0.3

$p < 0.01$ **

Table 3.7: The combination of cyclosporine with prednisone induced a 44% reduction in insulin sensitivity.

Overall there was not change in mean basal glucose observed in the above studies, while on therapy. However, 4 of 20 dogs became overtly diabetic while on therapy (1 with low-dose prednisone, 1 with high-dose prednisone, and 2 with combined prednisone and cyclosporine). It was noted that each of these four animals had very poor baseline islet functional reserve ($K_G \leq 1.0$ % per min. in all cases). In these instances, diabetes was irreversible despite withdrawal of therapy and temporary treatment with insulin. It was not possible to complete FSIGT's on these animals in the presence of overt diabetes.

3.4.4 TACROLIMUS:

Tacrolimus monotherapy did not significantly alter K_G (figure 3.7), but there was a strong trend towards reduction of insulin sensitivity (p=0.06, table 3.8). Interestingly, one month of tacrolimus therapy (level controlled to 10-15 µg/L) given to normal (non-transplanted) control dogs (n=6) led to a 44% decline in K_G, which was significant ($p < 0.01$, figure 3.7),

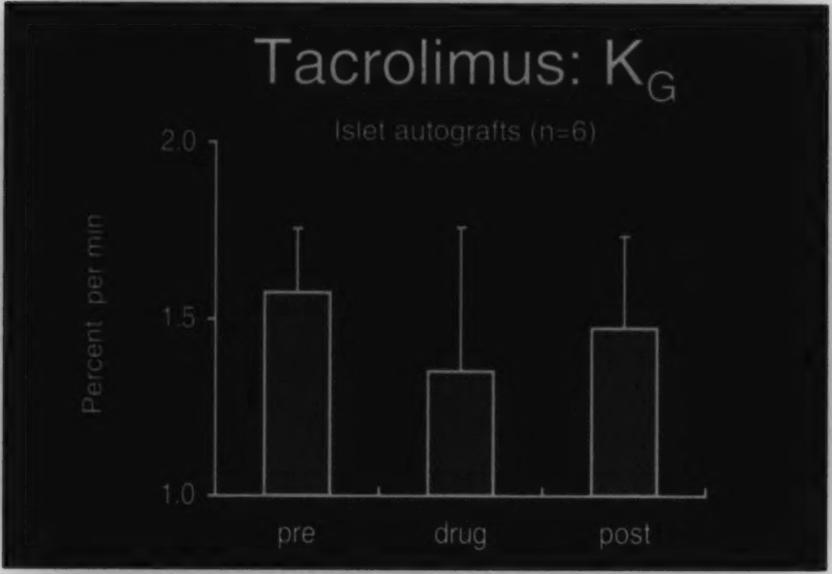
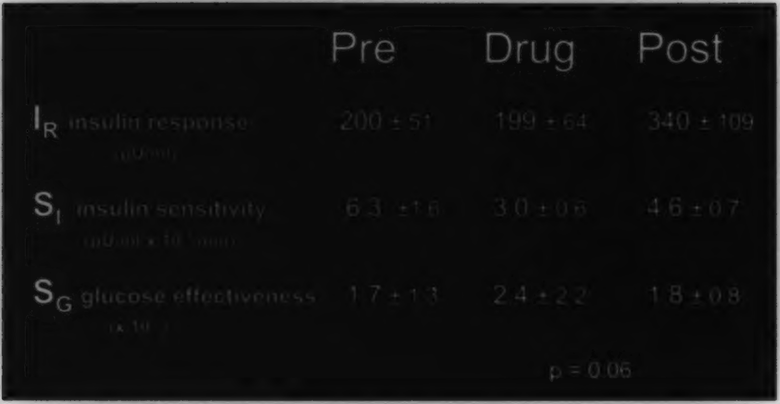


Figure 3.7: Impact of tacrolimus monotherapy on canine islet autograft recipients – did not lead to significant impairment in K_G

Tacrolimus



	Pre	Drug	Post
I_R insulin response	200 ± 51	199 ± 64	340 ± 109
S_I insulin sensitivity	6.3 ±1.6	3.0 ± 0.6	4.6 ± 0.7
S_G glucose effectiveness	1.7 ± 1.3	2.4 ± 2.2	1.8 ± 0.8

$p = 0.06$

Table 3.8: Impact of tacrolimus monotherapy on canine islet autograft recipients – reduced insulin sensitivity

3.4.5 TACROLIMUS AND GLUCOCORTICOIDS:

The administration of tacrolimus and low-dose prednisone led to irreversible and permanent islet autograft failure in ALL recipients (n=6, p<0.001) within 7-21 days of therapy. FSIGT data was not obtained since all of the recipients were overtly diabetic, and was therefore felt to be superfluous in this situation. Glucose decay data (K_G) were therefore obtained on a series of 6 healthy control dogs before, at one month of therapy, and after a further month after withdrawal of tacrolimus/prednisone therapy. Results are illustrated in figure 3.8 below. Endogenous pancreatic islet function deteriorated by 44% while on therapy ($p < 0.01$), and did not return to baseline after withdrawal of therapy.

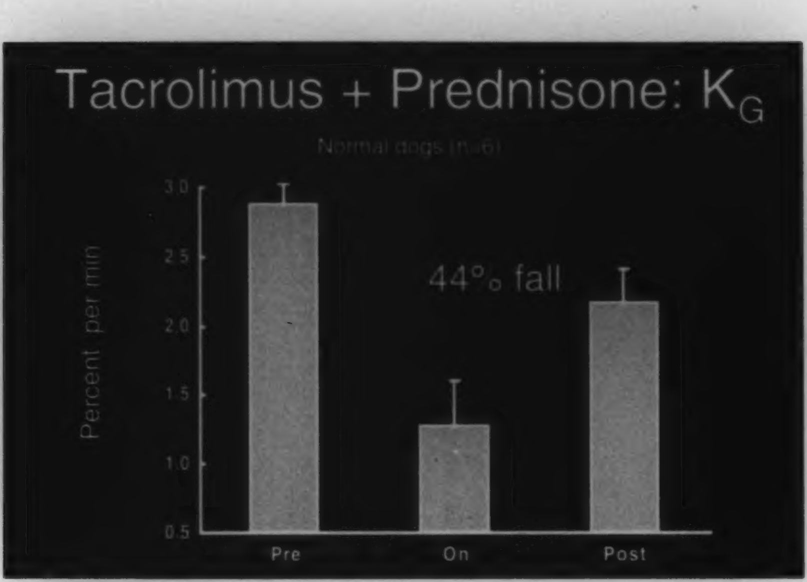
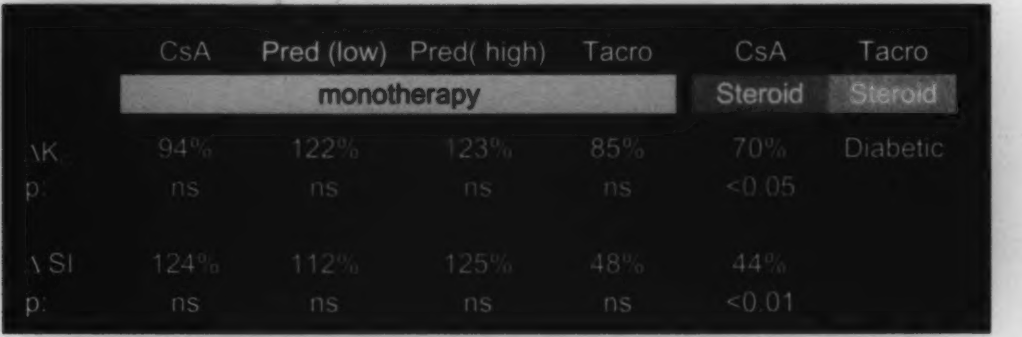


Figure 3.8: Impact of low dose prednisone + tacrolimus (level controlled to 10-15 µg/L given to NORMAL control dogs led to a significant impairment in endogenous pancreas islet function, with 44% decline in K_G.

3.4.6 SUMMARY OF FINDINGS:

A summary of impact of calcineurin inhibitor and combination glucocorticoid therapy on islet autograft function as defined by percent change in KG and SI while on therapy compared with baseline, is provided below (table 3.9):



	CsA	Pred (low)	Pred(high)	Tacro	CsA	Tacro
	monotherapy				Steroid	Steroid
\K	94%	122%	123%	85%	70%	Diabetic
p:	ns	ns	ns	ns	<0.05	
\ SI	124%	112%	125%	48%	44%	
p:	ns	ns	ns	ns	<0.01	

Table 3.9: Synopsis of experimental data, expressed as percent change in glucose decay (K_G) or insulin sensitivity (SI)

3.5 DISCUSSION

Design of optimal immunosuppressive regimens for clinical islet transplantation dictate that therapy be sufficiently potent to prevent both destruction from acute or chronic rejection and also recurrence of autoimmune diabetes. Up till recently, the only available effective immunosuppression was diabetogenic – being directly toxic to beta cells or promoting peripheral insulin resistance. Avoidance of toxic immunosuppression has been proposed as one explanation for the success of islet auto-transplantation in patients undergoing total pancreatectomy for chronic pancreatitis, together with the fact that islets were implanted in a non-immune environment(*3, 36*). Of 447 islet allografts performed in patients with Type 1 diabetes, fewer than 10% were able to discontinue insulin for longer than one year, where cyclosporine, glucocorticoids and azathioprine were used as combined maintenance immunosuppression in the majority of cases(*2, 12*). More recent data suggests that with optimal islet mass, up to 50% of patients may have evidence of ongoing graft function, but less than 20% of patients achieved insulin independence with cyclosporine-glucocorticoid based therapy(*13-15*)

Kneteman *et al* were the first to demonstrate detrimental effects of prednisone on canine islet autograft function in 1987(*37-39*). These finding that were confirmed subsequently by Kaufman, Zeng and others in canine islet autograft and allograft studies(*9, 11*). The first clinical trial of steroid avoidance in islet transplantation was reported by Ricordi *et al* in patients undergoing cluster islet-liver replacement after abdominal exenteration where high dose tacrolimus monotherapy was used in some cases – and may have been one of the main factors securing the high rate of insulin independence reported in that trial(*40, 41*).

In the current study, one month of therapy with cyclosporine monotherapy did not influence K_G or other parameters of autograft function, confirming previous studies in the attempts to implant human autografted islets into the spleen have not only met with poor functional outcome, but have in some cases resulted in major morbidities including splenic rupture, gastric perforation and venous thrombosis(*43, 44*).

Kneteman *et al.* previously addressed the metabolic impact of sirolimus and CsA after chronic administration in dogs bearing intrasplenic islet autografts(*27*). FSIGT's performed after one month of sirolimus monotherapy revealed significant improvement in K_G, explained in part though an increase in basal and stimulated insulin secretion. Insulin clearance was also reduced by 13% in these studies(*27*). When sirolimus was given in combination with CsA, the direct metabolic benefit of sirolimus was less, but the elevated total insulin response to glucose persisted.

The current studies showed that glucocorticoid monotherapy has minimal impact on the function of engrafted islet autografts in an intrasplenic heterotopic site, except in those grafts with marginal baseline insulin secretory reserve ($K_G \leq 1.0$ percent per min). Cyclosporine or tacrolimus monotherapy also demonstrated minimal impact on islet autograft function. The combination of glucocorticoid and calcineurin inhibitor caused marked impairment in islet autograft function in cyclosporine-treated recipients, but caused complete and irreversible graft failure in the tacrolimus-treated group. The glucocorticoid-tacrolimus combination was also found to be highly diabetogenic to endogenous islet function in healthy non-transplanted dogs. The Minimal Model data suggested that the major diabetogenic effects were mediated through a marked reduction in peripheral insulin sensitivity, which was not completely reversible on cessation of therapy. The combination of tacrolimus with sirolimus was not tested in this model because preceding therapy with tacrolimus-glucocorticoid – an outcome that was unexpected from previous studies, destroyed all long-term islet autografts.

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These studies reiterate a critical need to tailor immunosuppression to the specific needs of an islet transplant; a renewed opportunity has been provided by the promising array of new immunosuppressive therapies that may offer the future potential for both calcineurin-inhibitor and glucocorticoid-free protocols.

3.6 ACKNOWLEDGEMENTS

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CHAPTER 4:

THE PORTAL IMMUNOSUPPRESSIVE STORM – PORTAL AND SYSTEMIC PHARMACOKINETICS OF NEORAL, SANDIMMUNE, TACROLIMUS AND SIROLIMUS AFTER ORAL THERAPY

NOTE: A previous version of this chapter is currently under review in *Transplantation*, and authors on this paper include:
Shapiro AMJ, Gallant HL, Hao EG, Wong J, Lakey JRT,
Rajotte RV, Yatscoff RW and Kneteman NM.

4.1 ABSTRACT

Background: Experimental and clinical evidence suggests that liver transplants are less prone to rejection-mediated graft loss than other organs under comparable immunosuppression, but the underlying protective mechanisms remain conjectural. Local high dose immunosuppressant delivery via the portal vein may be one contributing factor. The purpose of this study is to compare acute and steady state pharmacokinetic profiles of a panel of immunosuppressants in portal and systemic blood after oral absorption.

Methods: Six healthy mongrel dogs (20-25kg) underwent placement of chronic subcutaneously tunneled portal vein and carotid artery catheters. A five-day course of oral drug was then given in random sequence, with a two week rest between agents (Neoral^{TM} vs Sandimmune (5mg/kg/bid); Tacrolimus (0.075mg/kg/bid); Sirolimus (2.5mg/kg/d).

Results: C_{max} for acute Sandimmune (mean ±sem) was 2686 ±449 (portal) vs 1183 ±131µg/L (systemic) ($p<0.05$, 3-way ANOVA). The AUC_{4h} was 5219 ±870 (portal) vs 3219 ±400µg/L (systemic) ($p=0.002$). Steady-state pharmacokinetics (day 5) were significantly higher for Neoral^{TM} in portal vs systemic blood than for Sandimmune (7466 ±980 Neoral^{TM} portal vs 5433 ±950µg/L Sandimmune portal, $p=0.01$). Similar profiles were obtained for Tacrolimus and Sirolimus.

Conclusions: Peak immunosuppressant levels, as well as early and chronic area-under-the-curve are dramatically elevated in portal blood for all drugs tested. This change is accentuated with Neoral^{TM} micro-emulsion compared with Sandimmune. The enhanced portal immunosuppressive drug availability may be an important explanation for enhanced efficacy of Neoral^{TM} when used without sequential intravenous CsA. The

'portal storm' of local immunosuppression is an important phenomenon, and may have particular bearing on liver (enhancement) and intrahepatic islet transplantation (toxicity).

4.2 INTRODUCTION

Local high-peak immunosuppressant delivery via the portal vein may be one explanation for the fact that liver transplants are less prone to rejection-mediated graft loss than other organs under comparable oral immunosuppressive therapy(*1, 2*). The underlying protective mechanisms remain conjectural however, and may include: a) the unique property of the liver to regenerate and repair its constitutive cells after rejection-mediated injury(*3, 4*), b) that the macrolide calcineurin inhibitors are hepatotrophic(*5, 6*), c) the possibility that hepatocytes may be less immunogenic(*7*), d) portal antigen delivery and processing may confer additional immuno-protection(*8-10*), e) secretion of soluble antigens by the liver with innate capacity for potent systemic immunosuppressive effect(*11, 12*), and f) the effects of first-pass enterocyte and hepatocyte metabolism in altering the profile of immunosuppressive metabolites in portal compared with systemic blood(*13, 14*).

Kidney, pancreas, heart and lung transplants are exposed to systemic drug levels (and secondary metabolites), and may be potentially less protected, whereas an islet transplant, embolized to the liver via the portal vein, could be exposed to drug levels toxic for the beta-cell – at least in the early post-transplant phase before islet neovascularization is complete. This is reflected by the results of the Islet Transplant Registry, where less than 8% of over 447 diabetic allograft recipients immunosuppressed with cyclosporine-based therapy were able to sustain insulin-independence for periods longer than one year(*15-17*). Results from selected centers reported occasional success in islet-kidney recipients immunosuppressed with glucocorticoid and cyclosporine-based therapy, including previous results from Edmonton (1989 – 1994), where 2 of 7 patients were insulin independent at one year, one of whom remained insulin free for over 2 years post transplant. Only recently have the results of clinical islet transplantation improved substantially; development of more potent but less diabetogenic immunosuppression has been a key factor in this progress, with the use of a glucocorticoid-free protocol based on daclizumab, sirolimus and low-dose tacrolimus (Prograf^{TM}), with the latter drug given at 75% reduced dose from that used previously in transplantation(*18-20*).

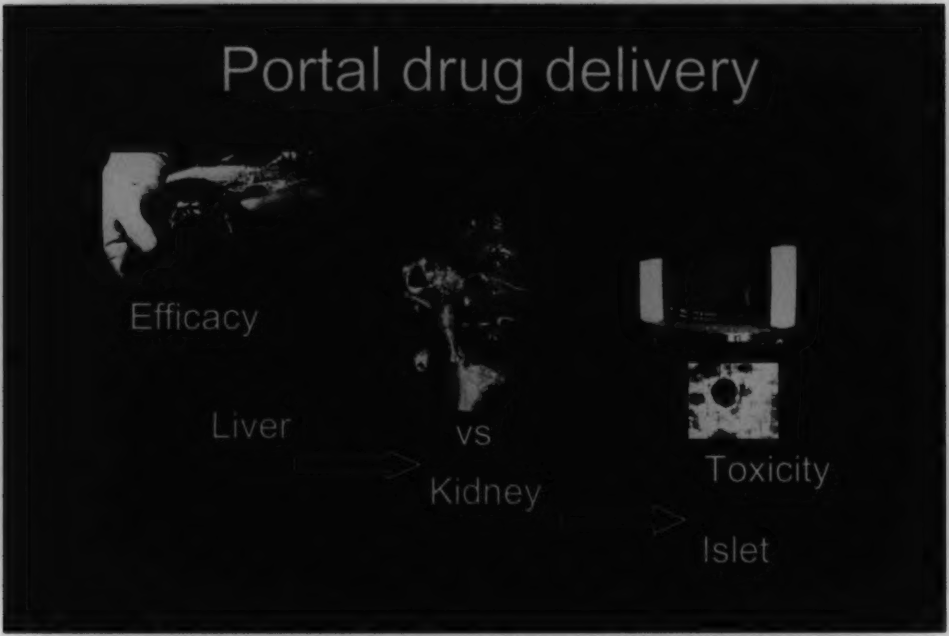
The concept of local graft immunosuppression is an attractive one, and is based on the expanded therapeutic index of enhanced local efficacy with reduced systemic toxicity. Extensive experimental evidence supports the concept that local immunosuppressant delivery can control immunologic events occurring at the graft site without relying entirely on systemic inhibition of lymphocyte activation(*21, 22*). Effector cells differentiate and mature within the allograft in a process that is dependent upon local production of lymphokines with local up-regulation of endothelial-lymphocyte interaction and diapedesis. Based on this concept, the local impact of immunosuppressive agents is likely to be substantial. The applicability of this approach has been limited in practice by technical difficulties in maintaining chronic local access catheters without increased risk of line infection, line occlusion or vessel thrombosis(*22*). Advances in genetic engineering have extended the concept to local production and/or secretion of immunosuppressive molecules such as CD40L, CTLA4-Ig, TGF-b or Fas-ligand, and experimental data in small animals offers promise that this approach may one day aid in achieving transplantation across major allogeneic or even xenogeneic barriers without the concomitant need for systemic immunosuppression(*23-28*)

A further phenomenon that has been difficult to explain is the inconsistency of decreased risk of rejection within the first 3 months in studies comparing Sandimmune cyclosporine with its replacement micro-emulsion formulation Neoral^{TM}. Hemming et al reported a decrease in rate of acute rejection from 75% with Sandimmune to 35% using Neoral^{TM} without intravenous cyclosporine in clinical liver transplantation by 3 months(*29, 30*). A Canadian multi-center study (NOF-8) comparing Sandimmune with Neoral did not demonstrate a reduced incidence of acute rejection, a finding thought possibly to relate to the use of intravenous cyclosporine during induction, although the newer formulation was clearly better absorbed and target drug levels were easier to attain(*31*). This study and others have clearly demonstrated the important potential of peak drug level monitoring (C_{max}) rather than trough level monitoring in the prevention of acute rejection, and high peak portal drug levels may therefore have particular bearing in liver transplantation(*30, 32, 33*).

In order to further characterize the above series of observations, we herein investigate a panel of immunosuppressive agents in portal and systemic blood after oral administration, to better define the pharmacokinetics of portal delivery of immunosuppressants to the liver.

4.3 HYPOTHESIS*:*

Oral immunosuppressant absorption leads to "a storm" of high peak drug delivery in portal blood, which is of potential benefit for "local" immunosuppression in liver transplantation, but is potentially harmful to an islet transplant embolized to an intra-portal site.



Portal drug delivery

Efficacy

Liver

vs

Kidney

Toxicity

Islet

Figure 4.1: Expressing the concept of the "portal storm", and its potential beneficial implications for liver transplantation, but potential detrimental impact on an engrafting islet transplant in the early phase post-implantation.

Left figure – human liver transplant ex vivo; middle – kidney transplant ex vivo; right – upper figure shows transparent isolation chamber; lower figure shows dithizone-stained human islet.

4.4 MATERIALS AND METHODS

Six healthy mongrel dogs of weight 20-25kg underwent placement of chronic subcutaneously-tunneled cuffed silastic catheters in the portal vein and carotid artery to permit pharmacokinetic blood samples to be withdrawn from awake, non-restrained animals. Silastic catheters were prepared using techniques modified from O'Brien et al(*34*). Briefly, two silastic catheters (0.062 inch internal diameter x 0.125 inch outer diameter) (Dow Corning, Midland, MI) were cut to length (70cm). Double velour Dacron cuffs (Meadox, Oakland, NJ) were cut to 2cm diameter discs and threaded to a distance of 15cm from the external end of each catheter. The discs were glued in place with medical grade silicone elastomer glue (Silastic, Dow Corning, Midland, MI), and fixed at a distance of 15cm from the external ends of the catheters. A further 4mm diameter lip of silicone glue was positioned at 10cm or 15cm from the internal tip of the portal or carotid catheter respectively. The purpose of the lip was to aid in securing the catheter within each vessel to prevent withdrawal beyond the level of ligation. Multiple side-holes of 1mm diameter were cut in the distal 2cm of the catheter tip to reduce the possibility of aspirating against the side-wall of the vessel during blood sampling. Catheters were then packaged and sterilized in ethylene oxide gas.

Anesthesia and surgery: After an overnight fast, dogs underwent endotracheal intubation and general anesthesia with halothane inhalation after intramuscular sedation with a mixture (0.1ml/kg) containing acepromazine (1mg), meperidine (12mg) and atropine (2.5mg) made up in 10mls of normal saline. Prophylactic antibiotic was administered on induction (cloxacillin 1g i.m.), and continued for 3 days post-operatively (cloxacillin 500mg i.m. qd). The right side and dorsal aspect of the neck, the right lateral flank, and midline abdomen were then shaved and prepared for surgery. A dorsal midline incision was made in the neck, and two subcutaneous pockets were made with blunt dissection to accommodate the Dacron catheter cuffs. A separate incision was made in the line of the right common carotid artery to expose and control this vessel, and after pre-flushing of the catheter with heparin-saline (100iU/ml), the artery was occluded proximally and distally, an arteriotomy made, and the catheter guided centrally towards the heart until the silicone anchor bead was reached. The catheter was then secured in place with 3 0/silk ties, permanently occluding the vessel to prevent bleeding or dislodgement at the entry site. The catheter was then aspirated and flushed to confirm satisfactory placement, then delivered externally via the dorsal neck site and capped with a luer-lock hub (PRN adaptor, Deseret Medical Inc., Sandy, UT). For placement of the portal catheter, the catheter was pre-flushed with heparin-saline (100iU/ml), delivered externally through the dorsal neck pocket, and tunneled with a 100cm tunneling device to a separate incision in the right flank. The latter incision was then deepened to the level of the peritoneum, a small hole in the peritoneum made, and the catheter threaded into the peritoneal cavity. The lateral incision was then closed in layers with 2/0 Vicryl (muscle) and 3/0 Vicryl (skin). A separate midline abdominal incision was then made, deepened through the linea alba, and the peritoneal cavity entered. After removal of the extraperitoneal fat pad using cautery, a self-retaining Balfour retractor was used to gain maximal exposure of the main portal vein. The gastroduodenal vein was used for catheter access to the portal vein, the catheter tip palpated just proximal to the portal bifurcation, and secured within the stump of the gastroduodenal vein just distal to the silicone catheter retention bead using 3/O silk. Before closing the abdomen the catheter was aspirated and flushed with heparin-saline to confirm satisfactory placement. The Abdomen was then closed *en masse* with running 0/prolene with reinforced 3/0 Vicryl subcuticular skin closure. After extubation, animals were transferred to heated cages. Analgesia was given as required using buprenorphine (0.1-0.2 mg/kg) (Schering, Toronto, Ont., Canada). Fluid balance was achieved intra-operatively with Ringer's lactate solution (75mls/hour i.v.) and by subcutaneous boluses post-operatively. Free access to water was provided on day 1, and a regular standard diet offered thereafter. A recovery period of 14 days then allowed animals to recover from the effects of surgery, and to regain normal bowel function. All animals were cared for by personnel of the Health Sciences Laboratory Animal Services of the University of Alberta under direct supervision of a veterinarian, and in compliance with guidelines of the Canadian Council on Animal Care. All animals were sacrificed humanely upon completion of the study.

Catheter maintenance: Catheters were flushed on a daily basis with 0.9% saline followed by a heparin-saline lock (1000iU/ml). Approximately 0.1ml excess heparin-saline than the catheter dead-space was given to maintain patency. After one week post-surgery, the heparin-saline lock concentration was increased to 10,000iU/ml to reduce the chance of catheter thrombosis. Catheters were cleansed every 3-4 days by replacing the PRN adaptors, aspirating the heparin-saline locks, instilling 0.9% saline followed by a sterilizing solution of ACD containing formaldehyde (0.4% anhydrous citric acid, 1.32% sodium citrate (dihydrate), 1.47% dextrose (mono H_20) and 1.5% formaldehyde) to fill the catheter dead-space. The solution was left in place for 5 minutes, aspirated, the catheter re-flushed with 0.9% saline and re-locked with heparin-saline. Non-constricting neck bandages were made to prevent the animals from damaging the external catheter tips.

Experimental design: After a recovery period of two weeks from surgery, dogs were randomized to receive either Sandimmune or Neoral™ for a period of five days by mouth. After 14 days without drug, animals were crossed over to receive the alternate cyclosporine preparation. Paired pharmacokinetic blood samples were drawn from portal and carotid cannulae on day 1 (after acute drug administration) and again on day 5 (after chronic, steady state half-lives had been reached). After a further 14 days, a smaller pilot study of 2 dogs were treated with Tacrolimus for 5 days, and the remaining 4 dogs were treated with Sirolimus for 5 days.

Drugs and administration: Sandimmune and Neoral™ Cyclosporine were given in capsule form, as supplied by Novartis Pharmaceuticals Canada Inc., at a clinically relevant dose of 5mg/kg/bid. Tacrolimus was also given in capsule form (a gift from Fujisawa Canada) at a dose of 0.075mg/kg/bid. Sirolimus liquid (a gift from Wyeth-Ayerst Research, Princeton, NJ) was given at a dose of 2.5mg/kg/day.

Pharmacokinetic studies: Paired 5ml blood samples were drawn from portal and carotid cannulae at time 0, 0.5, 1, 2, 4, 6, 8 and 12 hours after oral dosing. Catheters were flushed with 0.9% saline between sampling, and a 10ml blood aspirate was drawn prior to each sample and replaced immediately thereafter to ensure that there was no sample contamination or dilution occurred due to the catheter dead-space. Whole-blood samples were stored at 4°C in EDTA tubes and drug levels were processed on the following day. Parent cyclosporine drug levels were analyzed by fluorescence polarization immunoassay (FPIA, Abbot TDX, Abbot Inc., Abbot Park, IL, USA) and confirmed by HPLC(*35, 36*). Parent Tacrolimus levels were assayed by a specific monoclonal antibody ELISA (Incstar Inc., Stillwater, MN, USA). Sirolimus drug levels were analyzed by reverse-phase HPLC, as described by Yatscoff *et al.*, and has a sensitivity of 0.5µg/L with an intra-assay coefficient of variation of 15% and 10% at 10µg/L and 50µg/L respectively(*37*).

The following pharmacokinetic parameters were measured by standard techniques; maximal concentration (C_{max}), time to C_{max}, (t_{max}), area under the concentration-time curve from 0 to 12 h (AUC_{12h}), area under the concentration-time curve from 0 to 4 h (AUC_{4h}) and terminal half-life ($t_{1/2}$).

Statistical analysis: Results are expressed as mean ± sem, and statistical comparisons made using the t-test for paired data or the three-way analysis of variance (ANOVA) with Tukey post-hoc comparison as appropriate (Sigmastat version 2.0 for Windows 95), with $p < 0.05$ being considered statistically significant. Pharmacokinetic parameters were calculated using WinNonlin software (version 1.0), using the trapezoid rule for calculation of AUC.

4.5 RESULTS

4.5.1 COMPARISON OF SANDIMMUNE VS NEORAL™ FORMULATIONS:

The 12-hour pharmacokinetic profiles of Sandimmune and Neoral™ after acute administration are shown in figure 4.2 below.

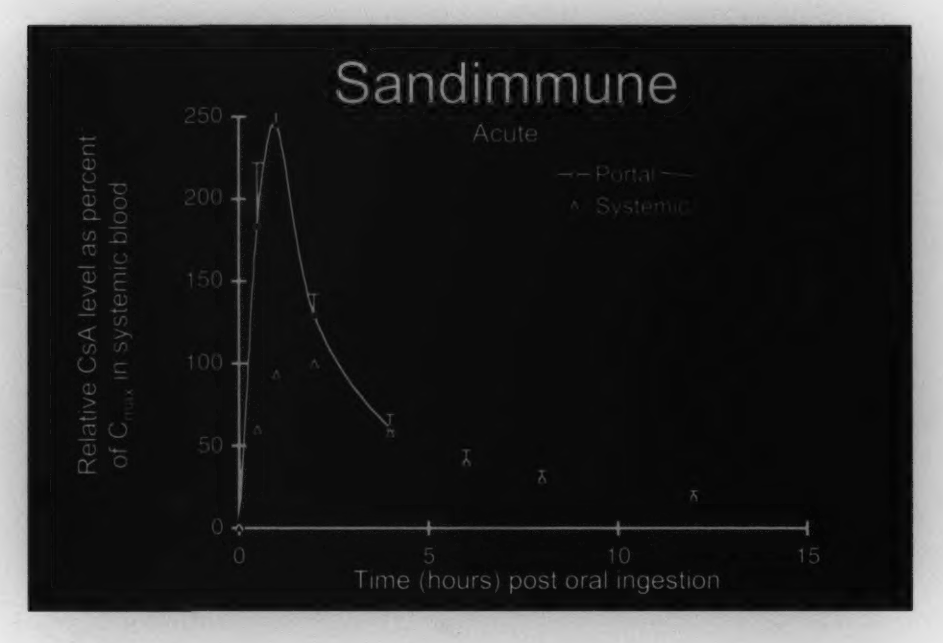


Figure 4.2: Cyclosporine pharmacokinetic drug levels in portal (white) and systemic (pink) blood (mean ± sem, n=6 per group) after acute oral administration of Sandimmune (dose 5mg/kg bid PO; levels measured by HPLC; values are expressed as a percent of peak levels in systemic blood)

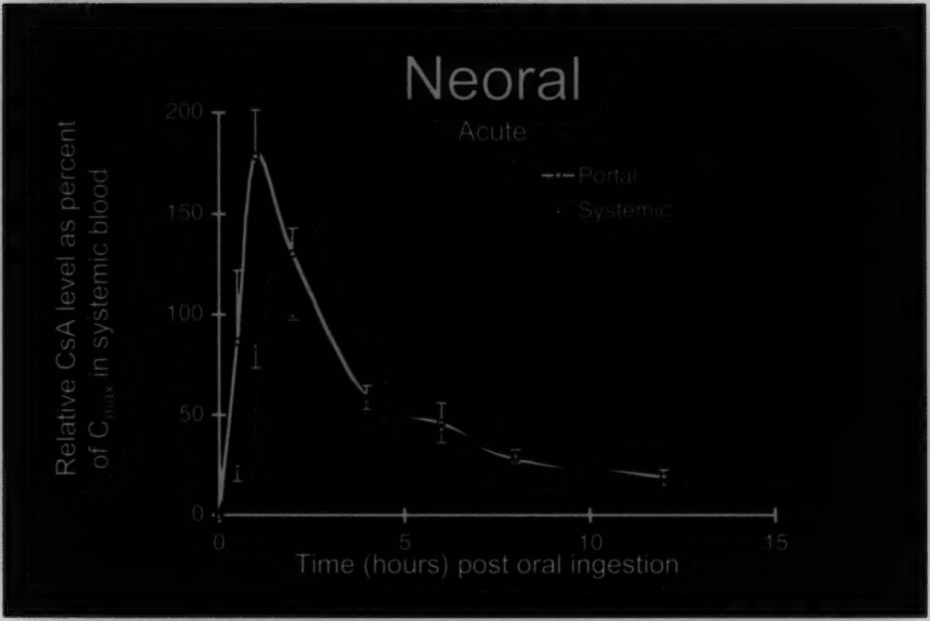


Figure 4.3: Cyclosporine pharmacokinetic drug levels in portal (white) and systemic (pink) blood (mean ± sem, n=6 per group) after acute oral administration of Neoral. (Note: values are expressed as a percent relative to peak level measured in systemic blood).

Figure 4.2 and 4.3 compare paired mean ± SEM cyclosporine levels for Sandimmune and Neoral™ respectively in portal and systemic blood, with values expressed relative to the C_{max} in systemic blood, in order to correct for variability in drug absorption between dogs.

C_{max} values for Sandimmune and Neoral™ were 227% ±49 ($p = 0.015$) and 182.9% ± 23 ($p = 0.009$) higher in portal blood compared with systemic values respectively after acute oral therapy. Furthermore, mean trough systemic cyclosporine levels of 213.7 ±39 µg/L for Neoral™ correspond with C_{max} values of 2656 ± 330 µg/L in portal blood ($p < 0.001$), representing a difference in excess of 12 times in magnitude. Figure 4.4 compares absolute mean ± sem Cyclosporine levels for Neoral™ in portal and systemic blood, and portal and systemic data for steady state peripheral intravenous infusion of cyclosporine (3mg/kg/day) is shown for further comparison.

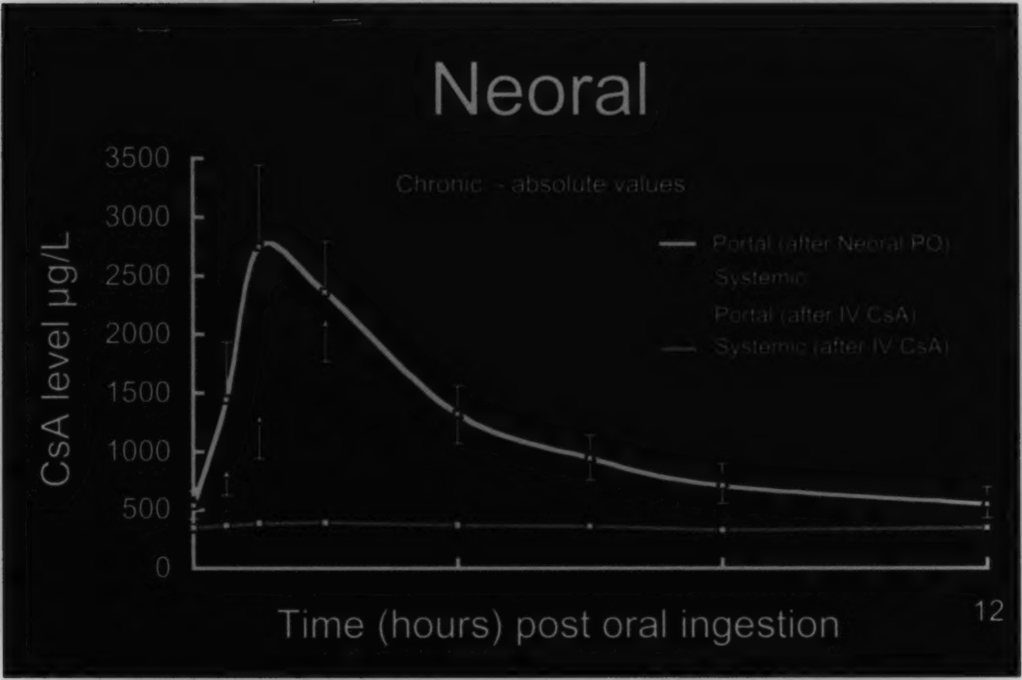
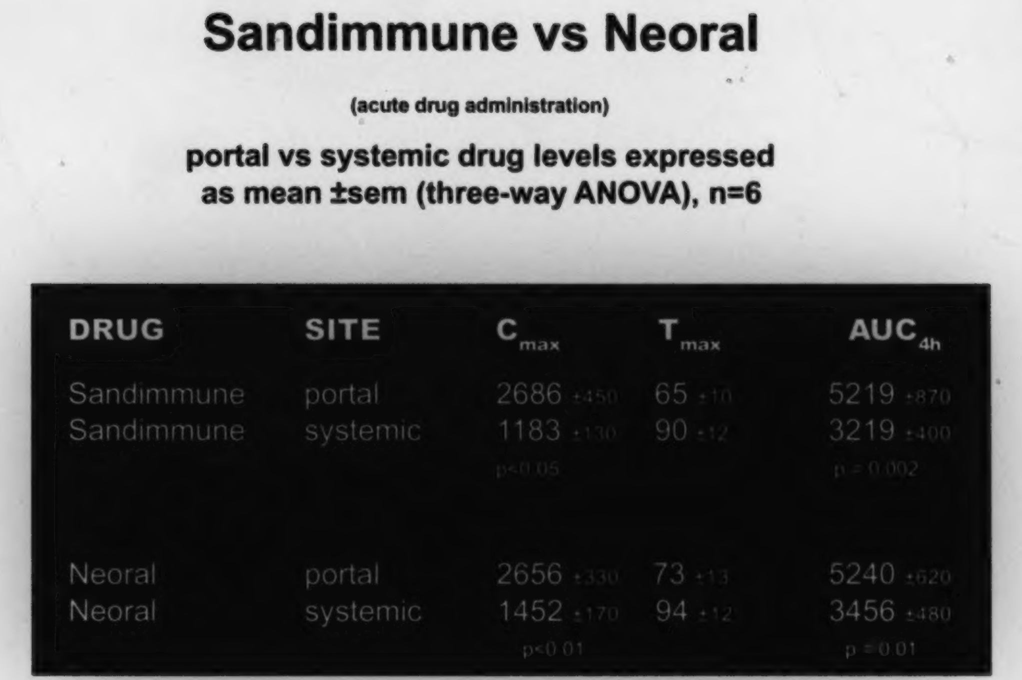


Figure 4.4: displays ABSOLUTE Cyclosporine levels in portal (white), systemic (pink) after oral Neoral therapy, compared with portal (blue) and systemic (orange) levels after steady-state IV CsA therapy (µg/L TDX assay,*mean ±SEM*).

Table 4.1 summarizes the pharmacokinetic data for acute administration of Sandimmune and Neoral™ (see below). The AUC_{4h} for Sandimmune and Neoral™ were 162% and 152% higher in portal blood compared to systemic values (p = 0.002 and p = 0.01 respectively), representing substantial elevation in early drug exposure in portal blood. There was a trend towards earlier T_{max} in portal blood both for Sandimmune and Neoral™, but this did not achieve statistical significance.



Sandimmune vs Neoral

(acute drug administration)

portal vs systemic drug levels expressed as mean ±sem (three-way ANOVA), n=6

DRUG	SITE	C_{max}	T_{max}	AUC_{4h}
Sandimmune	portal	2686 ±450	65 ±10	5219 ±870
Sandimmune	systemic	1183 ±130	90 ±12	3219 ±400
		$p<0.05$		$p = 0.002$
Neoral	portal	2656 ±330	73 ±13	5240 ±620
Neoral	systemic	1452 ±170	94 ±12	3456 ±480
		$p<0.01$		$p = 0.01$

Table 4.1: Summary of pharmacokinetic data for Sandimmune vs Neoral (µg/L TDX assay, *mean ±SEM*) (acute administration)

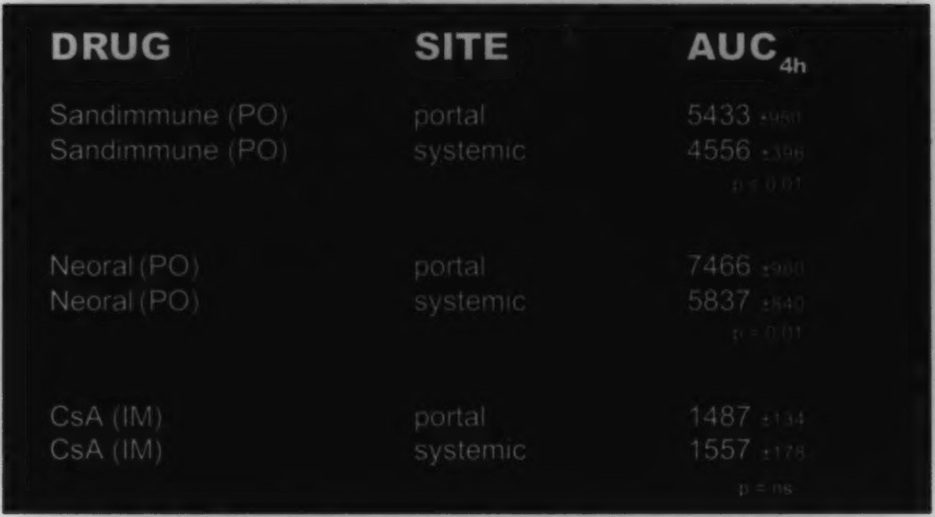
Table 4.2 summarizes the pharmacokinetic data for chronic (steady state) administration of oral Sandimmune and oral Neoral^{TM}.

The portal-systemic difference in AUC_{4h} for Neoral^{TM} after chronic administration was substantially higher than for Sandimmune. In a three-way ANOVA for source of variation of independent variables, pharmacokinetic differences in AUC_{4h} comparing Neoral^{TM} vs Sandimmune were highly significant (p=0.003, power = 0.99, a=0.05), as were differences between AUC_{4h} portal and systemic drug exposure (p=0.02, power = 0.74, a=0.05). Differences in inter-subject variability were also independently significant (p < 0.001, power = 1.00, a=0.05).

Sandimmune vs Neoral

(chronic drug administration)

portal vs systemic drug levels expressed as mean ±sem (three-way ANOVA), n=6



DRUG	SITE	AUC_{4h}
Sandimmune (PO)	portal	5433 ±950
Sandimmune (PO)	systemic	4556 ±396
		$p \le 0.01$
Neoral (PO)	portal	7466 ±900
Neoral (PO)	systemic	5837 ±840
		$p = 0.01$
CsA (IM)	portal	1487 ±134
CsA (IM)	systemic	1557 ±178
		p = ns

Table 4.2: Summary of pharmacokinetic data for Sandimmune vs Neoral (chronic) (µg/L TDX assay, *mean ±SEM*).

4.5.2 TACROLIMUS:

Figure 4.5 and 4.6 demonstrate ELISA drug levels for Tacrolimus in portal and systemic blood, with Figure 4.5 referring to acute and Figure 4.6 referring to chronic drug administration.

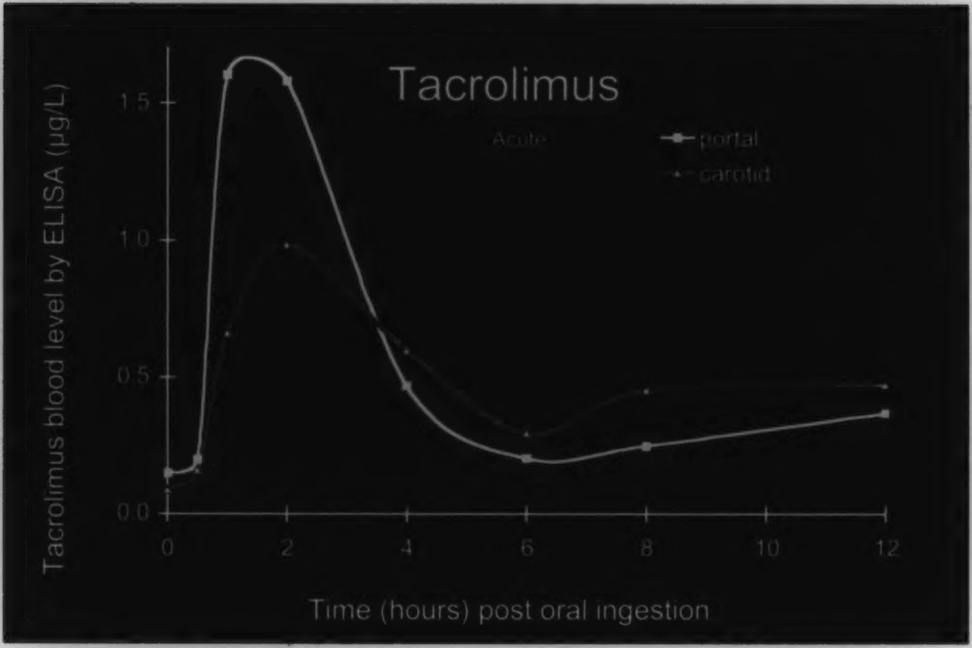


Figure 4.5: Tacrolimus pharmacokinetic drug levels in portal (white) and systemic (orange) blood after acute therapy *(dose 0.075mg/kg twice daily PO). (Tacrolimus levels are in µg/L by ELISA, mean ±SEM).*

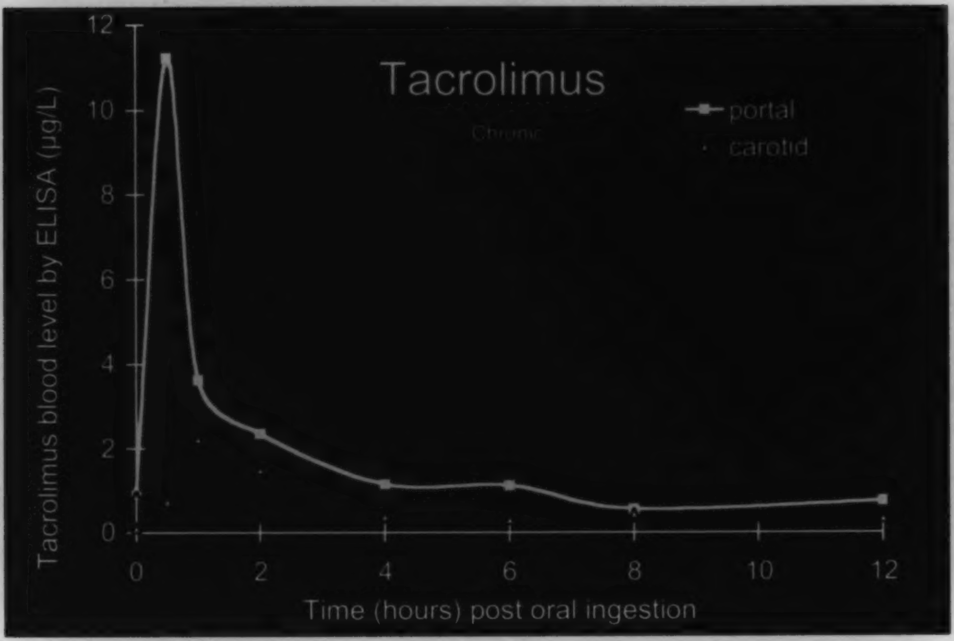


Figure 4.6: Tacrolimus pharmacokinetic drug levels in portal (white) and systemic (orange) blood after chronic therapy.*(Tacrolimus levels are in µg/L by ELISA, mean ±SEM).*

Although limited numbers in this arm of the study do not permit detailed statistical comparison, it is evident that portal-systemic differences in C_{max} and AUC_{4h} for Tacrolimus correspond closely to the patterns seen with Cyclosporine. This difference is even more striking after chronic drug exposure.

4.5.3 SIROLIMUS:

Figure 4.7 shows drug levels for Sirolimus in portal and systemic blood after acute oral therapy, and demonstrates highly significant elevation in portal drug levels (p = 0.001).

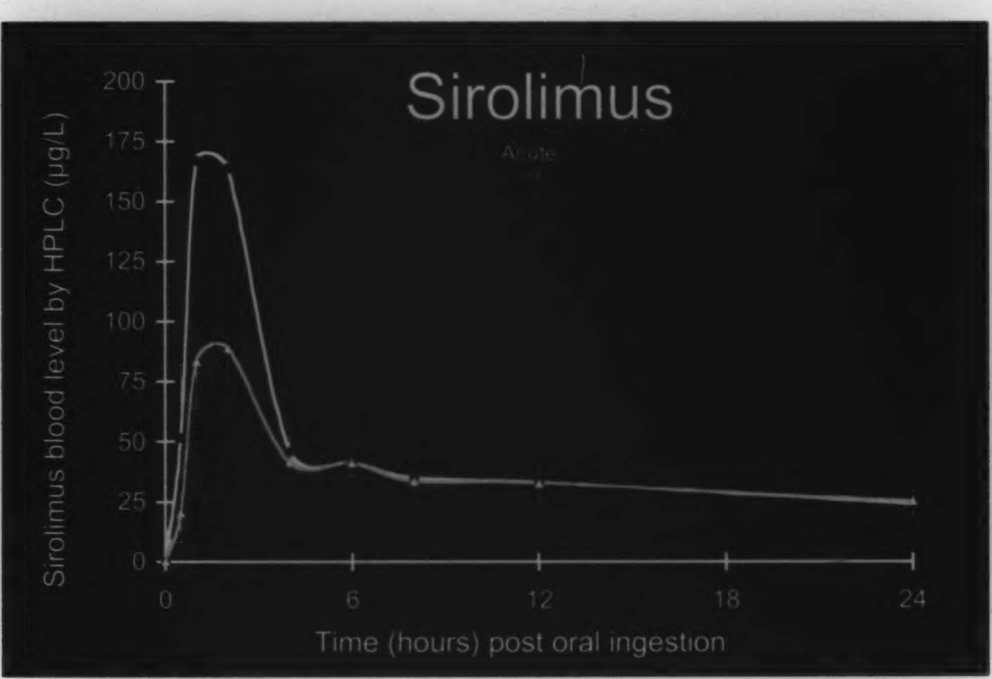


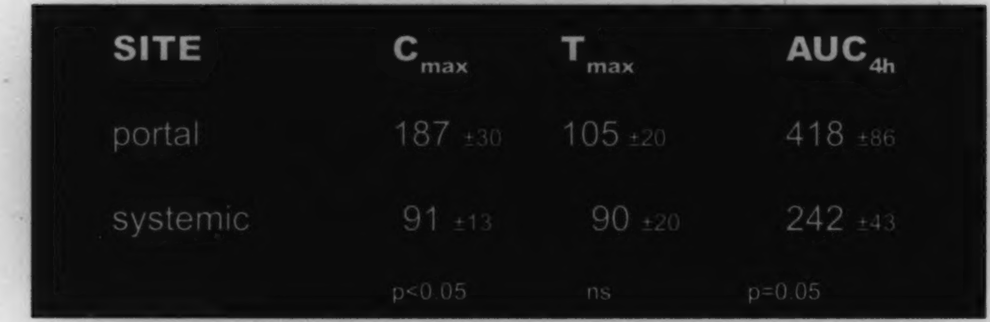
Figure 4.7: Sirolimus pharmacokinetic drug levels in portal (white) and systemic (yellow) blood after acute therapy. *(Sirolimus dose 2.5mg/kg/day PO; levels are in µg/L by HPLC, mean ±SEM).*

Table 4.3 summarizes the pharmacokinetic analyses for Sirolimus (see below). Similar again to the pattern seen with Cyclosporine, the C_{max} was 205% higher in portal blood ($p < 0.05$), and the AUC_{4h} was 173% higher in portal blood ($p = 0.05$), representing marked elevation in early exposure to Sirolimus in portal blood.

Sirolimus

(acute administration)

portal vs systemic drug levels expressed as mean ± sem (three-way ANOVA), n=4



SITE	C_{max}	T_{max}	AUC_{4h}
portal	187 ±30	105 ±20	418 ±86
systemic	91 ±13	90 ±20	242 ±43
	$p<0.05$	ns	$p=0.05$

Table 4.3: Summary of pharmacokinetic data for Sirolimus *(Sirolimus levels are in µg/L by HPLC, mean ±SEM).*

4.6 DISCUSSION

The pharmacokinetics of immunosuppressants in portal blood have been characterized in only a limited number of studies to date. Freeman *et al* showed that AUC and C_{max} for Sandimmune was higher in portal blood in the pig compared with hepatic venous or jugular venous blood(*38*). Gridelli et al found similar results after a single dose of Sandimmune in the dog(*39*). Detailed comparisons of portal and systemic pharmacokinetics were not made in either of these studies, and we are not aware of similar portal pharmacokinetic studies comparing Sandimmune with Neoral™, or characterizing the profiles of Tacrolimus or Sirolimus. Based on the current and above supporting data, it appears that the "portal immunosuppressive storm" is clearly an established phenomenon. The question remains – what is its significance and how can it be harnessed to the benefit of transplantation?

Current results of clinical liver transplantation have improved dramatically, with one-year patient and graft survival expected in the non-emergency setting of 93% and 90% respectively(*1*). Many factors account for this success, including significant advances in surgical technique and more balanced use of immunosuppression, and as a result of this, rejection-mediated graft loss has become a rare event. Stable long-term function can be expected in the majority of cases, and a single episode of acute rejection does not correlate with impaired long-term outcome(*40, 41*). The liver enjoys immunological privilege compared to other organs, and although the incidence of acute rejection is similar, the liver is relatively resistant to antibody-mediated rejection, the incidence of chronic rejection is low, and episodes of acute and even chronic rejection are easily reversed in most cases. Indeed, the estimated half-life of a liver grafted in the most recent five years under tacrolimus immunosuppression has been estimated to be

15 to 20 years. The same is not true of kidney transplantation, where a single episode of acute rejection correlates strongly with late graft loss(*42, 43*). Despite significant improvement in the one-year outcome of kidney transplantation, it is surprising that expected improvements in mean kidney graft half-life, ranging from 6.5 to 11 years, have not followed suit over the past decade(*42, 44-46*). The "direct immunosuppressive hit" from the portal storm in favoring the outcome of liver over renal transplantation may contribute to this effect.

The comparative data from Sandimmune and Neoral™ in the current study confirm that peak, early and chronic AUC drug exposure is dramatically increased in portal blood. After steady-state pharmacokinetics are reached, the AUC_{4h} for Neoral™ is substantially elevated in portal blood compared with Sandimmune (7466±980 vs 5433±950μg/L/h, $p<0.01$), indicating accentuated drug exposure with the newer micro-emulsion formulation of Cyclosporine. Given that peripheral i.v. Cyclosporine infusion results in low steady-state levels in portal and systemic blood, the current study suggests that use of i.v. Cyclosporine without oral therapy in the critical early post-transplant period after liver transplantation may compromise efficacy by a loss of high peak, a lower area under the curve, and with a consequential loss of the markedly enhanced portal blood levels which would be seen by immune cells in the liver after hepatic transplantation. This may explain in part why clinical liver transplant studies of Neoral™ without intravenous Cyclosporine have demonstrated an expected reduction in incidence of acute rejection, whereas other studies have not(*29, 30*). An appreciation of the portal immunosuppressant pharmacokinetic data will take on particular relevance as clinical studies focus on the importance of peak rather than trough immunosuppressant level monitoring; preliminary clinical studies indicate that acute rejection events correlate more strongly with low peak levels rather than low troughs in peripheral blood(*30, 32, 33, 47-49*).

The poor results of clinical results of islet transplantation reported up till the introduction of a recent islet-specific low diabetogenic protocol(*20*), contrast sharply with the apparent success of this procedure in pre-clinical models of diabetes. The reasons for this are likely multifactorial, and reflect a limited engraftment islet mass, lack of a supporting exocrine stroma, the insulin-resistant state of the end-stage uremic diabetic, an inability to prevent, detect and treat early islet rejection, and use of immunosuppressants which are directly toxic to β-cell function. It is pertinent to note that the majority of large animal studies of long-term islet transplant function have been carried out in the dog, with islets infused into the spleen and where they would be shielded from the portal storm. The favored clinical site of implantation has been embolization directly to the liver via the portal vein. The peak portal drug levels (C_{max}) for the calcineurin inhibitors in the current study were in excess of 2,600μg/L for Cyclosporine preparations, and the peak levels for Tacrolimus in portal blood were in excess of ten times the peripheral 12-hour trough concentration. *In vitro* studies of islets exposed to immunosuppressants have shown that Cyclosporine levels exceeding 1,500μg/L or Tacrolimus levels above 10-100μg/L cause vacuolization and destruction of islets in culture (*50-52*). Similar changes have been identified in biopsies taken of long-term clinical whole pancreas allografts(*53*). Based on our data, with 12-hour peripheral trough levels above 300μg/L for Cyclosporine, or exceeding 10μg/L for Tacrolimus would certainly enter the toxic range for the β-cell. These calculations do not take into account any further detrimental effects these immunosuppressants may have in inducing peripheral insulin resistance, which would further impair control of glucose homeostasis.

It is difficult to predict what detrimental effect the portal immunosuppressive storm might have on late islet graft function after intra-portal embolization. Initial exposure to immunosuppressants in portal blood would be high during the initial 10-14 days while islet angiogenesis and neovascularization occurs, but is likely to become less over time, as remodeling ensues(*54-57*). Situated in direct proximity with the hepatocyte, there is presumably an immunosuppressant concentration gradient that could have persistent detrimental effects upon the islet long after the neovascularization process is complete. Indeed, recent studies by Wang et al comparing intra-portal vs peripheral intravenous continuous infusions of Tacrolimus have demonstrated that the immunological benefit of local intra-portal immunosuppression extends beyond the period of islet revascularization, and it is reasonable to assume that toxicity from local high peak immunosuppression would have a similar effect(*58*). The advantage of continuous low-dose local intra-portal infusion of Tacrolimus, avoiding the portal immunosuppressive storm of oral drug delivery, was of clear benefit for islet transplantation in this small animal study. It would be technically possible to deliver an identical local intra-portal immunosuppressive regimen in clinical islet transplantation, at least in the early post-transplant period.

In conclusion, the peak and early drug exposure of Sandimmune, Neoral^{TM}, Tacrolimus and Sirolimus are substantially higher in portal compared to systemic blood after oral therapy, accounting for the "portal immunosuppressive storm." This functional form of local immunosuppression may have bearing upon the apparent immunological privilege of liver transplantation, and may account in part for the limited previous success of clinical islet transplantation where islets are embolized intra-portally to an intra-hepatic site. A greater understanding of this concept may further allow development of oral immunosuppressant delivery systems tailored to meet the specific immunosuppressive needs of a particular organ.

4.7 ACKNOWLEDGMENTS

We would like to acknowledge the help and advice of D. W. O'Brien in the preparation of chronic portal and systemic cannulae for this study. Novartis Pharmaceuticals, Canada, generously donated Sandimmune and NeoralTM cyclosporine preparations. Tacrolimus (Prograf^{TM}) was kindly supplied by Fujisawa Canada, and Sirolimus (Rapamune) was a gift from Wyeth-Ayerst Research, USA. AMJS was supported by a Clinical Fellowship Award, and NMK by a Scholarship Award, both from the Alberta Heritage Foundation for Medical Research. This study was funded in part by a juvenile Diabetes Foundation International – Diabetes interdisciplinary Research Project grant.

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CHAPTER 5:

NOVEL APPROACHES TOWARD EARLY DIAGNOSIS OF ISLET ALLOGRAFT REJECTION

NOTE: A previous version of this chapter is currently in press in Transplantation, and will be published in the September 2001 issue. Authors on this paper include:
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5.1 ABSTRACT

Background: The inability to diagnose early rejection of an islet allograft has previously proved to be a major impediment to progress in clinical islet transplantation. The need to detect early rejection will become even more relevant as new tolerance-inducing protocols are evaluated in the clinic. We herein explore three novel approaches toward development of early diagnostic markers of islet rejection following islet allo-transplantation.

Methods: a) Canine islet allograft transplant recipients were immunosuppressed for one month then therapy was withdrawn. Serum glutamic acid decarboxylase antigen (GAD_{65}), an endogenous islet protein, was monitored daily with a CO_2 release assay. b) Rodent islets were genetically engineered to express a unique foreign protein (b-galactosidase) using adenoviral vectors, and after allograft transplantation, the viral-specific protein was measured in serum using optical luminescence. c) Rodents receiving islet allografts were immunosuppressed temporarily, and daily glucose tolerance tests were followed until graft failure occurred.

Results: a) Although serum monitoring of GAD_{65} antigen demonstrated elevated levels preceding loss of graft function in preliminary studies, the effect was not reproducible in all animals. b) Genetically engineered rodent islets demonstrated normal insulin kinetics in vitro (insulin stimulation index 2.57 ±0.2 vs. 2.95 ±0.3 for control islets, p=ns), and purified viral protein products had a stable half-life of 8 hours in vivo. After islet allo-transplantation, there were two peak elevations in serum viral proteins, confirming that an intra-islet "sentinel signal" could be detected serologically during acute rejection. There was no lead-time ahead of hyperglycemia, however. c) Daily sequential intravenous glucose tolerance tests demonstrated evidence of allograft

dysfunction (decline in K_G) with a two day lead time to hyperglycemia (2.58±0.3 vs. 1.63±0.2 %/min respectively, $p<0.001$), with an accuracy of 89%, sensitivity of 78% and specificity of 95%.

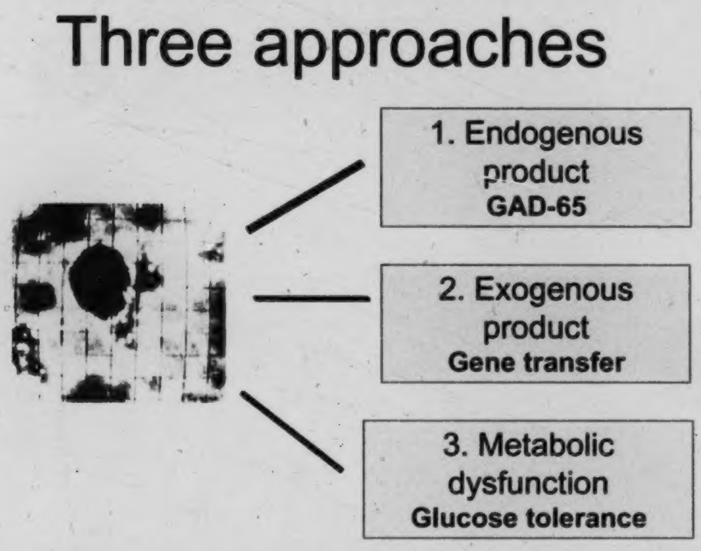
Conclusions: Of the three diagnostic tests, metabolic assessment with an abbreviated IVGT was the most effective method of demonstrating early islet dysfunction due to rejection.

5.2 INTRODUCTION

The inability to diagnose early rejection of an islet allograft has been a key factor that up till recently had severely hampered progress in clinical islet transplantation. This was a major drawback, as without recourse to appropriately directed intensification of immunosuppressive treatment in the background of rejection rates of up to 40-60% under glucocorticoid and cyclosporine immunosuppression, the majority of islet grafts were destroyed by their first and final rejection episode. In other forms of transplantation, a tissue biopsy would have provided confirmatory indication for intervention and reversal of rejection, following elevations in serum tests (e.g. liver enzymes for liver transplant, creatinine for kidney transplant, amylase, lipase and/or anodal trypsinogen for pancreas transplant). If solid organ transplants were thus handicapped, the current one-year graft survival in excess of 90% enjoyed by selected centers would fall dramatically, and would be predictably of the order of 40-60%(*1*).

Recent dramatic progress in clinical outcome of islet transplantation has enhanced previous graft survival from 8% at one year(*2, 3*) to 100% with sustained insulin independence in a small consecutive series of seven patients undergoing islet-alone transplantation at our center(*4, 5*). These grafts continue to function, providing sustained normoglycemia without insulin with the longest follow-up currently 21 months, without evidence of acute rejection or autoimmune recurrence of diabetes to date in a series that has now been expanded to include 14 patients. This transformation in outcome occurred through the use of more potent but non-diabetogenic immunosuppression (the combination of sirolimus with low-dose tacrolimus, and a short induction course of an anti-IL2Ra antibody) in a completely glucocorticoid-free regimen, coupled with transplantation of an adequate islet mass exceeding 10,000 IE/kg. Although an urgent need for diagnostic tests to detect islet rejection may appear to have diminished with the introduction of more specific, potent and balanced immunosuppression, as new tolerance-inducing protocols are further evaluated, the need for effective markers of islet rejection may become more critical.

The endocrine beta-cell reserve of an islet graft provides great capacity for compensation when the beta-cell mass is eroded during early acute rejection, as the remaining b-cells are able to sustain insulin output in response to hyperglycemic challenge. The onset of hyperglycemia in the failing islet allograft is therefore an end-stage event, occurring when more than 90% of the graft has been destroyed, and heralds irreversible graft failure(*6*). The endocrine islet reserve was demonstrated by Finch *et al*, who found that after 95% pancreatectomy only 53% of rats developed hyperglycemia(*7*). The lack of other specific markers of islet rejection has forced islet research to focus on prophylaxis and prevention of rejection rather than on diagnosis and treatment thereof. However, the majority of promising techniques known to induce long-term function or even stable tolerance in rodents have been difficult to apply in large animals, and are clearly a long way from clinical application. We therefore focused on the more immediately practical issue of how to detect early islet rejection by exploring three novel approaches.



Three approaches

1. Endogenous product
GAD-65

2. Exogenous product
Gene transfer

3. Metabolic dysfunction
Glucose tolerance

Figure 5.1: Approaches towards earlier diagnosis of islet rejection

The first approach explores the concept of detection of an endogenous islet protein in serum as it is released during the earliest phases of immune-mediated graft injury. Glutamic acid decarboxylase 65 (GAD_{65}) is a 65-kDalton polypeptide consisting of 585 amino acid residues, and is found ubiquitously only in pancreatic islets and in cells of the central nervous system(*8*). GAD is a pyridoxal phosphate- dependent enzyme that catalyzes the alpha-decarboxylation of glutamate to yield 4-amino-butyrate and CO_2, and is one of a series of islet auto-antigens shown to be a major target of humoral autoimmunity in type I diabetes(*9-11*). It exists in islets in two different inducible isoforms (GAD_{65} and GAD_{67}, the former being the only form found in human islets) that are stimulated by hyperglycemia, and it has been proposed that this may further accelerate b-cell destruction in diabetes(*12*). Monitoring of this islet specific protein seemed particularly relevant, since it has been shown that antibodies to GAD_{65} persist despite intensive immunosuppression, and may be associated with autoimmune recurrence and islet dysfunction after clinical islet transplantation(*13*).

The second approach is based on the genetic engineering of islets to express unique cytoplasmic proteins by means of an ultra-sensitive assay that can be detected in serum during early rejection. The underlying hypothesis is that insertion of a "sentinel signal" directly within islet cells will cause release of the unique product in serum during early rejection. For potential clinical applicability, any putative exogenous "sentinel signal" must: a) have neutral metabolic effect upon b-cell function, b) offer neutral or preferably enhanced immuno-protection, c) provide long-term stable expression, and d) have no risk of malignant transformation. In the near future, it is anticipated that transgenic pig islets or cloned human b-cells may fulfill this potential, but in the interim we herein tested the concept using adenoviral vectors. Using *in vitro* transduction with Ad5-cytomegaloviral b-galactosidase (b-gal), it has been shown recently that high levels of b-gal activity and viral mRNA can be detected histologically for more than 20 weeks after transplant, and that isografts have no evidence of viral-mediated destruction(*14, 15*). Furthermore, it has been possible to prolong islet allograft survival in rodents and in large animals by adenoviral or cationic transfer of various genes (bcl-2, adenovirus E3, catalase, HSP70)(*16-18*). Biolistic islet transformation using the "gene gun" is an alternative approach, and Gainer *et al* have recently shown extended murine islet allograft survival with transfected CTLA4Ig or soluble Fas-ligand using this technique(*19*).

The third approach in this paper utilizes serial metabolic challenge of islet graft function by intravenous glucose tolerance (IVGT) testing. The principle of glucose challenge by IVGT is to define maximal insulin secretory capacity, the surrogate of this being reflected by the glucose clearance rate (K_G)(*20*). While rejection-mediated elevation in basal glucose is a terminal event for the graft, it is anticipated that serial metabolic challenge will uncover earlier dysfunction that cannot be compensated for physiologically by remnant beta-cells.

5.3 MATERIALS AND METHODS

5.3.1 ENDOGENOUS MARKER OF REJECTION – SERUM GAD_{65} MONITORING

Experimental design: A total of 8 mongrel dogs (20-25kg) were used for this study. Autograft controls (n=2) underwent total pancreatectomy and intra-splenic islet autotransplantation without immunosuppression. Canine islet allografts (n=6) underwent total pancreatectomy and intra-splenic islet allo-transplantation, with temporary immunosuppression given for one month (CsA level controlled at 300µg/L, and Sirolimus at 0.05µg/kg IM). The canine islet transplant studies were carried out by Yakimets *et al* and transplant outcomes have been reported previously(*21*). Serum glucose was monitored daily, and GAD_{65} samples collected on alternate days until graft failure or until termination, and serum samples were stored at –70ºC for batch analysis. The dogs were maintained under the care of a veterinarian, and were housed and cared for in accordance with the recommendations of the Canadian Council on Animal Care.

Anesthesia and surgery: After on overnight fast, dogs underwent endotracheal intubation and general anesthesia with halothane inhalation after intramuscular sedation with a mixture (0.1ml/kg) containing acepromazine (1mg), meperidine (12mg) and atropine (2.5mg) made up in 10mls of normal saline. Prophylactic antibiotic was administered on induction (cloxacillin 1g i.m.). A total pancreatectomy was performed through an upper midline incision, preserving vascular supply to the duodenum.

Isolation, purification and transplantation of canine pancreatic islets: The autograft and allograft pancreata were prepared and processed according to methods described previously(*22, 23*). In brief, the pancreatic ducts were canulated and perfused with collagenase (2mg/ml collagenase type V, Sigma Chemical Co., St. Louis, MO) prepared in Hank's Balanced Salt Solution prior to transfer to an automated shaking chamber for continuous digestion at 37°C(*22*). At the digestion end-point, confirmed by the number and quality of liberated islets after dithizone staining, the tissue was washed in Hank's Balanced Salt Solution containing 10% fetal calf serum at 4°C. Islets were then purified on discontinuous Ficoll density gradients, and islets from the upper two interfaces were combined, washed, and counted prior to re-suspension in Medium 199 supplemented with 10% fetal calf serum (FCS) and antibiotics. Fresh islets were then infused into branches of the splenic vein of the recipient(*24*). After extubation, animals were transferred to heated cages for 24 hours postoperatively. Analgesia was given as required using buprenorphine (0.1-0.2 mg/kg) (Schering, Toronto, Ont., Canada). Fluid balance was achieved intra-operatively with Ringer's lactate solution (75mls/hour i.v.) and by subcutaneous boluses post-operatively.

Post-operative monitoring of glucose and GAD_{65}: Serum glucose was monitored daily after transplantation (Beckman glucose analyzer, Fullerton, CA), with rejection of an established islet graft defined as onset of fasting hyperglycemia >11mmol/L. Dialyzed serum samples were then measured for GAD_{65} activity on alternate days after transplantation using a sensitive CO_2-release assay. GAD_{65} is a pyridoxal phosphate-dependent enzyme that catalyzes the alpha-decarboxylation of glutamate to yield 4-

amino-butyrate and CO_2. The enzymatic activity of GAD_{65} was assayed by measurement of the conversion of L- (1-^{14}C) glutamic acid (Amersham Amersham, UK) to $^{14}CO_2$, according to methods described by Baekkeskov and others(*25-27*). One enzyme unit is defined as 1μmol of product formed per minute at 37°C. Using a similar assay, Petersen et al showed that the GAD_{65} enzyme activity of islets incubated in 5mmol/l glucose was of the order of 18 - 22μU/islet equivalent(*28*).

5.3.2 EXOGENOUS REJECTION MARKER - GENETIC ENGINEERING WITH SENTINEL SIGNAL INSERTION

Experimental design: Isolated rat islets were transduced *in vitro* with adenoviral vectors for Ad5-cytomegaloviral beta-gal. Satisfactory viral transfer was confirmed by X-gal staining for viral product in transduced but not in control islets(*14*). The function of transduced and control islets was assessed *in vitro* by perifusion in low and high glucose media, to exclude the possibility of viral-mediated damage. The *in vivo* half-life of pure beta-gal enzyme was evaluated in healthy adult rats to confirm stability in serum, and to exclude the possibility of pre-formed antibodies to the viral product. Finally, a series of adenovirally transduced islet allografts (n=6) were performed in diabetic rodent recipients, with serum glucose and b-gal monitored on a daily basis.

Animals and induction of diabetes: Male inbred Wistar-Furth (WF) rats ($RT1^U$) of weight range 250 - 275g were obtained from Harlan Sprague-Dawley inc., Indianapolis, USA and used as islet donors. Allograft recipients of Lewis strain (Lew) ($RT1^1$) were rendered diabetic by a single intravenous injection of streptozotocin (65 mg/kg body weight) (Sigma, St. Louis, MO, USA). Rats were considered diabetic after non-fasting glucose levels were confirmed ≥ 18 mmol/L on 3 or more occasions prior to transplantation. Glucose monitoring was performed on capillary tail blood with a

Companion II" blood glucose meter (Medisense Canada Inc.) Rat husbandry was carried out in accordance with the standards outlined by the Canadian Council on Animal Care.

Anesthesia, surgery, islet isolation and transplantation: Donor rats were anesthetized with sodium pentobarbital (0.4 mg/g body weight) and the bile duct canulated for retrograde pancreatic distension with 20 ml collagenase (1mg/ml Sigma type V). A modified stationary digestion technique was developed for this project to maximize islet yield from the rat pancreas (refer to Chapter 5 appendix for methods paper relating to the stationary digestion technique) (*29*). Briefly, pancreata were digested for 35 minutes at 37°C, vortexed, washed and passaged through a 850μm filter, and purified on discontinuous Ficoll gradients prior to re-suspension in Medium 199 containing 10% FCS for counting. In allograft experiments, 2,500 islets of diameter ≥100μm were transplanted beneath the left renal capsule of diabetic recipients.

Adenoviral gene transfer in vitro: Islets were rinsed in Medium 199, counted in aliquots of 500 and transferred to 15ml tubes (Sarstedt Inc., USA), centrifuged (1,000 rpm for 2 min) and the supernatant aspirated. Islet pellets were then transferred to individual wells of a 24 well plate in preparation for viral exposure. The viral construct was E1-deleted, replication-deficient and adenovirus serotype-5 derived(*30*). The cytomegalovirus (CMV) b-gal vector encodes a nuclear localizing form of Escherichia coli b-gal under control of a human CMV promoter enhancer(*31*). Although purified Ad5-CMV beta-gal is replication-defective, all steps involving viral handling were carried out in level II biohazard containment, under a designated level II hood. Ad5-CMV b-gal viral stock solution (maintained at -70°C) was pre-thawed, diluted in 2,024μL Hams F-10 media (containing 2% fetal calf serum and 10^{-6}M hydrocortisone) and 500μl added to each well (in a ratio of 1μL per islet, and at a concentration of 2.5 x 10^3 plaque forming units per islet). Control islet pellets were also transferred to individual wells and mixed in 500µL of virus-free HAMS media. Each 24-well plate was incubated at 37°C (5% CO_2) for 60 minutes, then islets were transferred to 15ml tubes and washed three times in Dulbecco's modified Eagle's Medium (DMEM) (Gibco) containing 10% fetal bovine serum (FBS), in preparation for culture or transplantation.

Islet staining for beta-gal: Whole islets were washed in phosphate-buffered saline (PBS), fixed for 5 min at 18°C in 1% formaldehyde, 0.2% glutaraldehyde, and washed three times with PBS before overnight staining at 37°C with 5-bromo-4-chloroindolyl-b-D-galactopyranoside (X-gal, American BioOrganics), as described by Smith *et al* (*32*). No background staining was observed in control non-transfected islets using this stain.

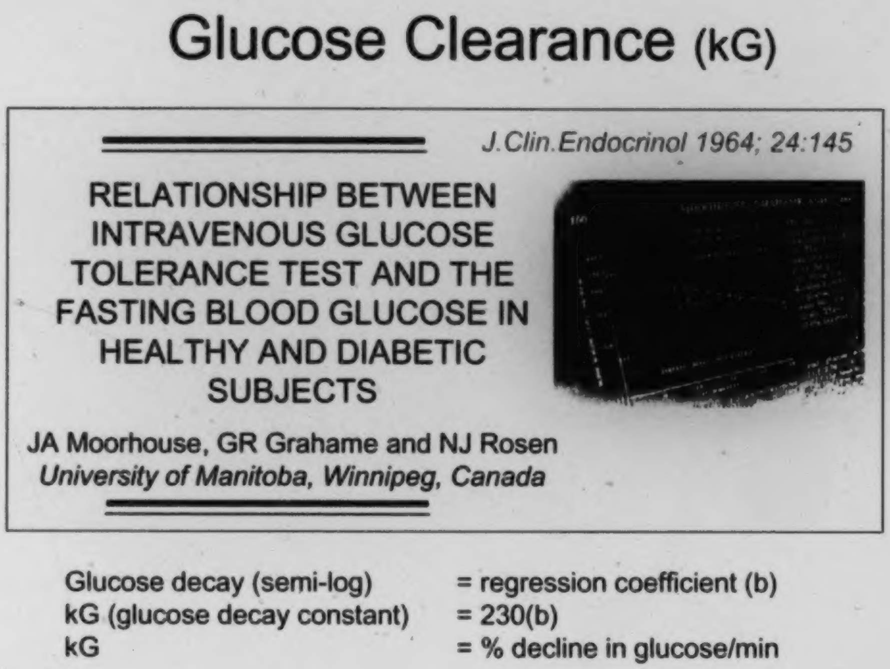
Serum assay for beta-gal activity, and assessment of half-life in vivo: A chemiluminescent assay system with light emission accelerator (Galacto-Light Plus™, Tropix) and tube luminometer were used for sensitive detection of beta-gal activity in serum (lower limit of detection 2fg of beta-gal (4000 molecules))(*33, 34*). The serum stability and half-life of pure beta-gal enzyme (1 unit per µl, Sigma) was determined *in vivo* by intravenous injection in healthy adult rats. One unit of pure beta-gal corresponded to 8.9×10^8 relative light units (RLU) in this assay.

Evaluation of islet function in vitro: Forty-eight hours after islet transduction and culture at 37°C, control and virally transduced islets were compared simultaneously *in vitro* by paired dynamic perifusion in low (2.8 mmol/l) and high (20 mmol/l) glucose media (200 islets per chamber, n=6 chambers), using the technique of Lacy et al(*35*). Insulin content was determined by double antibody radio-immunoassay (Pharmacia Diagnostics). The stimulation index (SI) (ratio of mean insulin release in high/low glucose media) and stimulated area-under-the-curve (S_{AUC}) were then calculated(*36*).

5.3.3 SERIAL METABOLIC MONITORING

Experimental design: A series of control islet isograft transplants (n=6, Group I) were performed in streptozotocin-diabetic WF rats, with graft function assessed by an abbreviated IVGT test on days 5 and 30 post-transplant. Islet allografts (n=10, Group II) were then performed under temporary immunosuppression, and serial IVGT tests were carried out daily from day 5 post-transplant until graft failure from rejection or experiment termination occurred.

Animals and the induction of diabetes: Rats of similar weight and source to experiment (b) above were used, and diabetes was induced with streptozotocin as above. WF strain rats were used as donors and isograft recipients; Lew strain rats were used as allograft recipients. *Islet isolation and transplantation:* Rodent islets were isolated using methods described in experiment (b) above(*29*). In this experiment however, the portal vein was chosen as the implantation site, with 3,000 islets embolized using an adapted 23G needle and syringe, under halothane inhalational anesthesia. The portal puncture site was sutured with 10-0 Prolene (Ethicon) with the aid of an operating microscope to reduce the risk of re-bleeding after placement of central lines and systemic heparinization. Baseline capillary glucose was monitored daily on tail blood samples after transplantation. Islet allograft recipients were given temporary immunosuppression with CsA (Novartis Canada), 20mg/kg/day by oral gavage from day 1 pre-transplant to day 3 post-transplant inclusive).



Glucose Clearance (kG)

J.Clin.Endocrinol 1964; 24:145

RELATIONSHIP BETWEEN INTRAVENOUS GLUCOSE TOLERANCE TEST AND THE FASTING BLOOD GLUCOSE IN HEALTHY AND DIABETIC SUBJECTS

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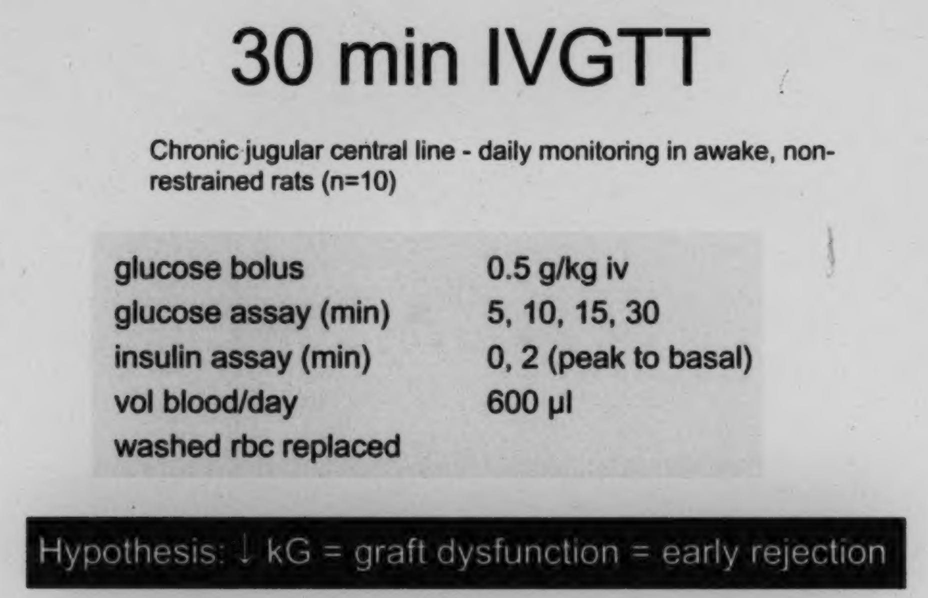
Glucose decay (semi-log) = regression coefficient (b)
kG (glucose decay constant) = 230(b)
kG = % decline in glucose/min

Figure 5.2: Original description of the glucose decay constant by Moorehouse.
(The inset graph (blue) demonstrates the regression constant and formula reported in the original paper)

The standard IVGT test, first described by Moorehouse *et al.*(*20*), was modified for daily assessment in the awake, non-restrained rat. A 3.5Fr silastic chronic central venous access catheter was placed in the right internal jugular vein under general anesthesia on the 5th post-transplant day, and tunneled to the back of the neck to prevent dislodgement. The catheter was flushed daily with 0.9% saline and locked with heparin-saline (1,000iU/ml) to maintain patency; occluded catheters were exchanged under general anesthesia if required. A 4-time-point IVGT was developed, with glucose samples drawn in sodium heparin tubes at 5, 10, 15 and 30 min post glucose bolus (0.5 g/kg iv).



30 min IVGTT

Chronic jugular central line - daily monitoring in awake, non-restrained rats (n=10)

glucose bolus	0.5 g/kg iv
glucose assay (min)	5, 10, 15, 30
insulin assay (min)	0, 2 (peak to basal)
vol blood/day	600 µl
washed rbc replaced	

Hypothesis: ↓ kG = graft dysfunction = early rejection

Table 5.1: Summary of abbreviated four time-point IVGTT

Further samples for basal and peak insulin output were drawn at 0 and 2 min after glucose injection. The total sample blood volume was 600 µl/day, and after centrifugation and aspiration of plasma, autologous washed red blood cells were auto-transfused daily to prevent anemia. Glucose samples were measured using a colorimetric single reagent glucose oxidase assay (Glucose Trinder, Sigma) and 96-well micro-plate reader (505nm filter), with the glucose disappearance constant (K_G) computed from a log-linear regression equation as previously described(*20, 37*). Plasma insulin was assayed by a double antibody radioimmunoassay (Pharmacia), and the *in vivo* stimulation index calculated (peak/basal insulin).

Statistical analysis: Results for all experimental groups are expressed as mean ± sem, and statistical comparisons made using the t-test for paired data, analysis of variance (ANOVA) for multiple comparisons, with Tukey *post hoc* analysis for parametric data. Non-parametric tests were carried out using the Kruskal-Wallis ANOVA, with Dunn's *post hoc* analysis as indicated. Computations were performed using Sigmastat version 2.0 for Windows 95, with $p < 0.05$ being considered statistically significant.

5.4 RESULTS

5.4.1 ENDOGENOUS REJECTION MARKER (SERUM GAD_{65} ACTIVITY)

Canine islet autograft controls (n=2) maintained normoglycemia throughout the study period. Serum GAD_{65} activity was not statistically different from background, as shown below.

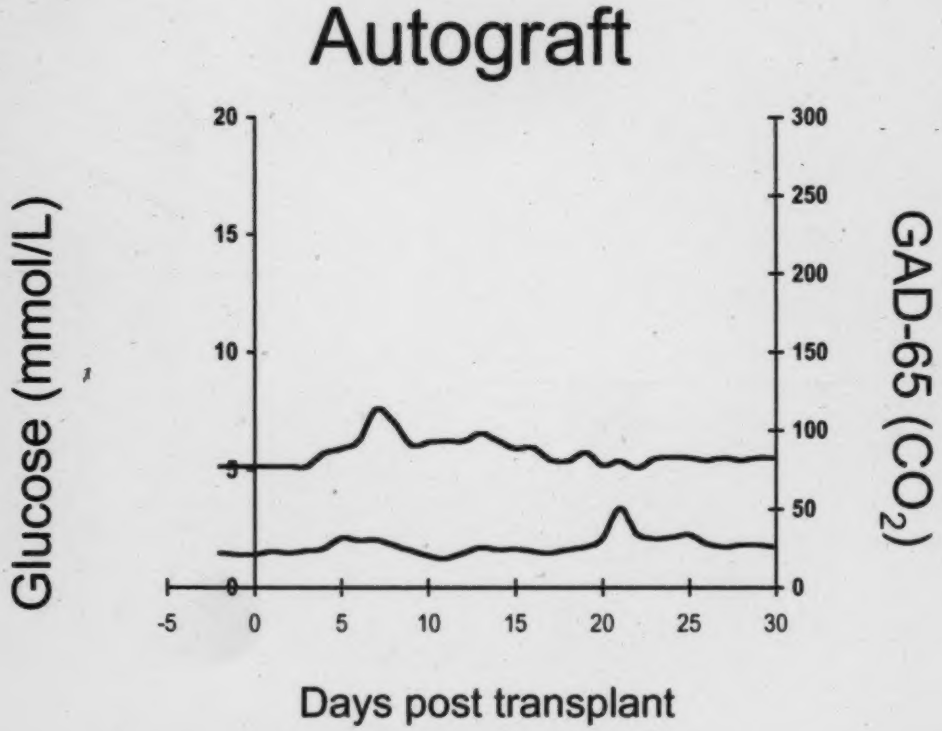


Figure 5.3: Stable levels of serum GAD-65 in canine islet autograft controls (n=2). Glucose (blue line) and GAD-65 (red line)

Canine islet allografts (n=6) became normoglycemic after transplantation but rejected on median day 17 (range 6-45), as evidenced by new onset of hyperglycemia.

Early acute rejection was strongly associated with CsA levels ≤ 250μg/l or Sirolimus levels ≤ 15μg/l, in accordance with previous studies from our laboratory(*21*).

One canine islet allograft recipient showed minor elevations in serum GAD_{65} activity occurring in synchrony with minor elevations in serum glucose during the first 30 days post-transplant. Five days after immunosuppressive withdrawal there were dramatic elevations in serum GAD_{65} activity that continued for 10 days until the eventual onset of hyperglycemia on day 45 post-transplant, as shown:

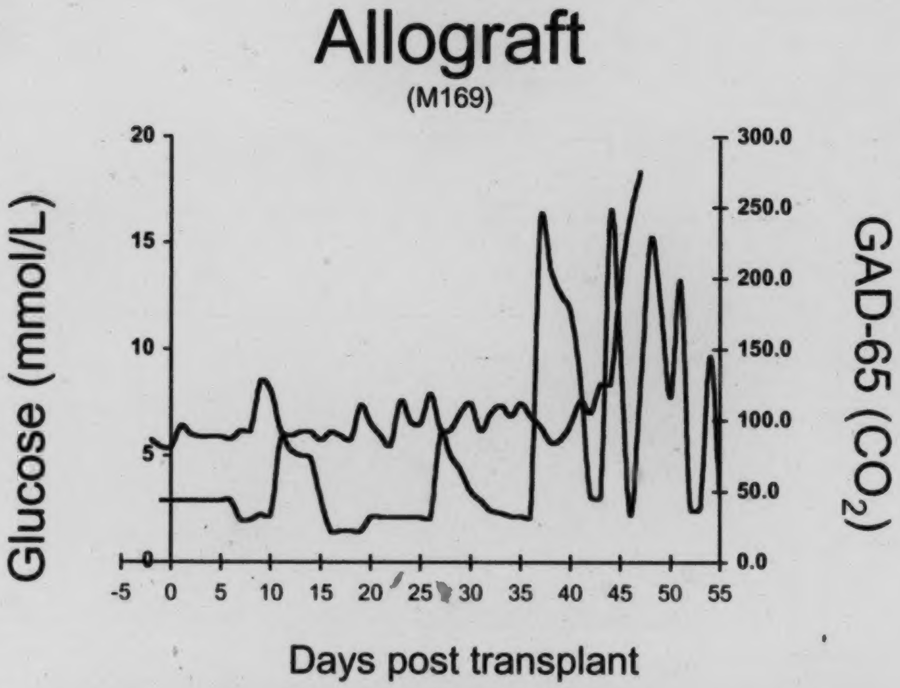


Figure 5.4: Perturbations in serum GAD-65 in canine islet allograft (M169) immunosuppressed temporarily with CsA and Sirolimus for 30 days.

Elevations in serum GAD_{65} activity occurred in synchrony with minor elevations in serum glucose during the first 30 days. Marked elevations in serum GAD_{65} activity occurred during the 10 days preceding the onset of hyperglycemia on day 45 post-transplant. This effect was not seen in the remaining 5 animals. Glucose (blue line) and GAD-65 (red line)

This effect was not found in the remaining 5 animals. Transient elevation in serum GAD_{65} activity was seen during the early engraftment period (days 1-2 post-transplant), but this did not reach significance (see Table 5.2 below):

Table 5.2: A summary of serum GAD_{65} activity in canine islet autografts and allografts (mean ±SEM).

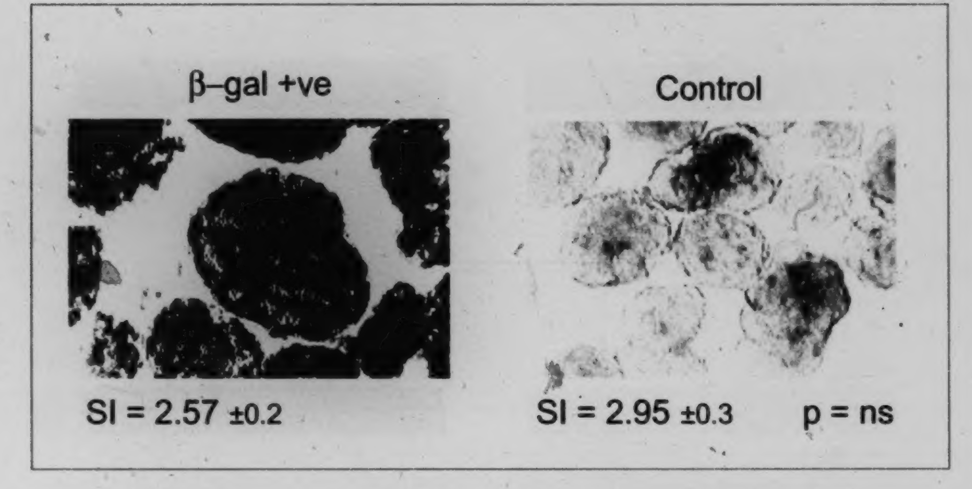
Although there was a trend towards higher mean serum GAD_{65} levels in the 5 days preceding rejection (51.8±12 vs. 32.5±1.9, mean ±sem, n=6), this also failed to achieve statistical significance.

5.4.2 EXOGENOUS REJECTION MARKER (SERUM BETA-GAL ACTIVITY)

X-gal staining of Ad-5 CMV beta-gal transduced WF islets revealed dense beta-gal activity after culture, whereas control non-transduced WF islets showed no evidence of background activity, confirming satisfactory gene transfer, as shown below:

β-gal

Successful gene transfer—YES
Evidence of islet toxicity—NO



β–gal +ve

Control

SI = 2.57 ±0.2

SI = 2.95 ±0.3 p = ns

Figure 5.5: X-gal staining of Ad-5 β-gal CMV transduced and control islets. The Stimulation Index (SI) of transduced (left figure) and control islets (right figure) *in vitro* demonstrate no evidence of islet toxicity (SI=Stimulation index).

On dynamic perifusion in high and low glucose solution, the mean SI and S_{AUC} for transduced vs. control islets were statistically similar (SI: 2.57±0.2 vs. 2.95±0.3 (p = ns) and S_{AUC}: $6.6 \pm 1.5 \times 10^3$ vs. $6.9 \pm 2.0 \times 10^3$ (p = ns) respectively), confirming that viral transduction did not impair islet insulin secretory capacity *in vitro*.

Intravenous injection of non-diabetic healthy WF rats (n=4) with purified beta-gal enzyme (20 units) demonstrated a predictable log-linear half-life, reaching background serum activity within 8 hours:

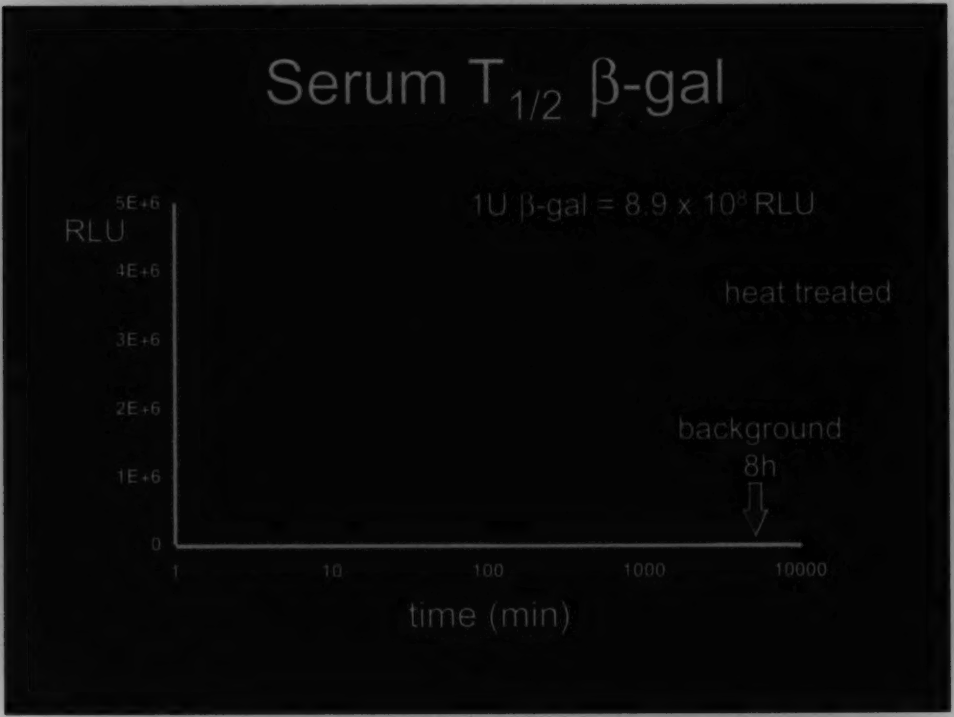
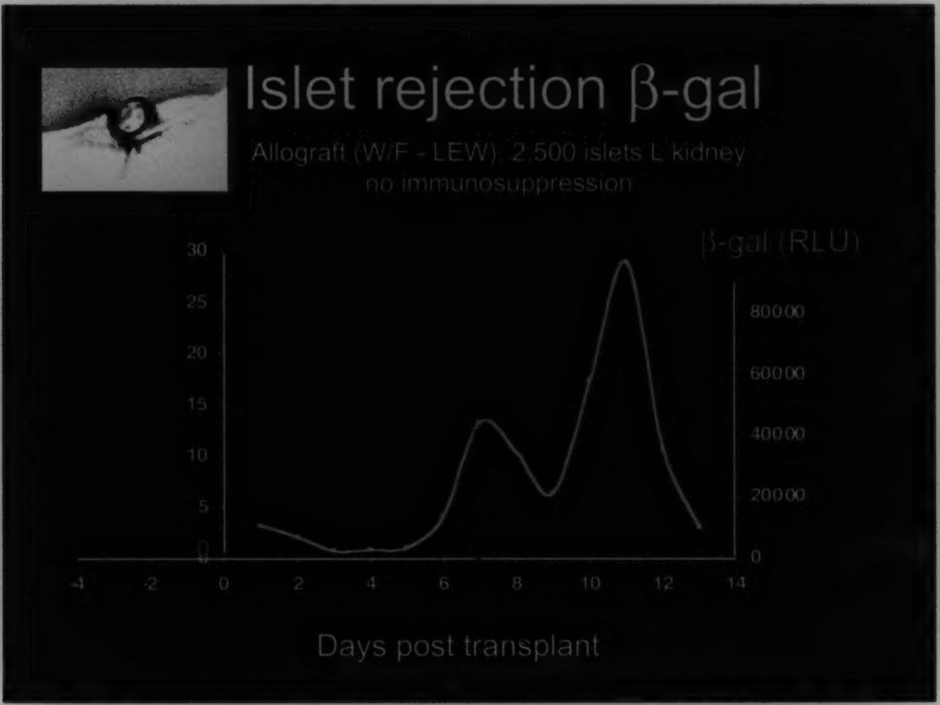


Figure 5.6: Serum β-gal activity – control data. The serum half-life of pure β-gal *in vivo*, and stability of this product in heat-treated and stored serum.

Half-life in vivo (red); half-life in vitro in fresh serum (blue) and heat-treated serum (brown)

There was no evidence of pre-formed antibody to beta-gal protein, and b-gal enzyme activity showed no sign of decay over time when incubated with either heat-treated or fresh rat serum. The Galacto-Light Plus™ chemiluminescent beta-gal assay therefore appeared robust for sensitive serum-monitoring, and had a co-efficient of variation <15%.

Islet allograft transplants in streptozotocin-diabetic recipients (n=6) restored normoglycemia in all cases, indicating that viral transduction did not impact islet insulin secretory capacity *in vivo*. Without immunosuppression, recipients became hyperglycemic uniformly by day 6, a pace of rejection that was statistically similar to our previous experience with this strain combination, indicating that viral transduction had not markedly increased islet immunogenicity. Serum b-gal activity was considerably higher than background during the initial phase of islet engraftment (by a factor of x 20), and rose substantially in a bi-modal pattern during islet rejection, peaking at a 180-fold increase compared to background:



Islet rejection β-gal

Allograft (W/F - LEW), 2,500 islets L kidney
no immunosuppression

Figure 5.7: Serum β-gal activity – transplant data. . Serum glucose (green line) and serum b-gal activity (yellow line) in rat islet allografts (n=6) after Ad-5 β-gal CMV transduction.

Based on the assay relationship of 1 unit beta-gal corresponding to 8.9 x 10^8 relative light units (RLU), the peak levels of 9.5 x 10^4 RLU, this represented detection of approximately 1.0 x 10^{-4} iU beta-gal diluted in 21cc of circulating blood volume, corresponding to a peak concentration of 2.0 x 10^{-3} iU b-gal per cc blood. Therefore even the relative small amounts of beta-gal released into serum were readily detectable at the time of rejection, and the bi-modal peak presumably reflected two phases of accelerated islet immune destruction with beta-gal release. However there was no lead-time detected for elevation in beta-gal compared to the onset of hyperglycemia, limiting the usefulness of this test as an early diagnostic marker of rejection.

5.4.3 SERIAL METABOLIC EVALUATION OF GRAFT FUNCTION (IVGTT)

Group I islet isograft controls (n=6) in non-immunosuppressed streptozotocin-diabetic recipients restored normoglycemia on the first post-operative day in all cases, which was maintained throughout the study period. Abbreviated IVGT tests conducted on day 5 and day 30 post-transplant revealed statistically similar results (day 5 K_G: 2.59±0.2 vs. day 30 K_G: 2.62±0.3, p = ns), indicating stable metabolic graft function over time.

In order to modify the pace of rejection and thus render the abbreviated IVGT test more clinically applicable, temporary immunosuppression was given by oral gavage to allograft recipients (CsA 20mg/kg from day –1 to +3 post transplant), which extended the median graft survival from 4.0 (4-5) to 9.0 (7-15) days (p<0.001) – see figure 5.8 below:

Delayed rejection

Short term CsA (20 mg/kg/day PO, day -1 to +3)

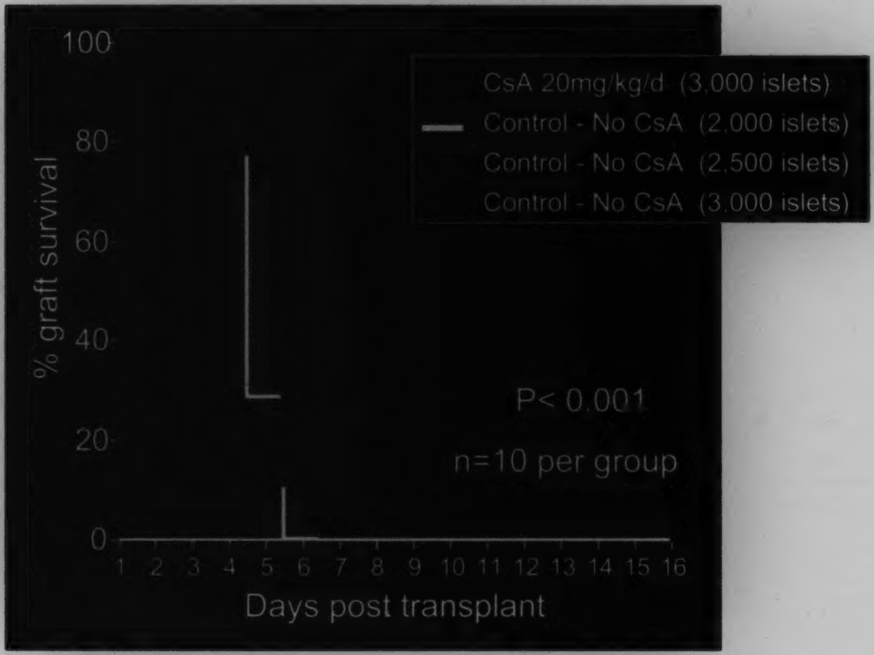


Figure 5.8: Temporary CsA treatment extended the median graft survival of islet allografts (WF into LEW) to 9 days, and reduces pace of rejection to enhance clinical relevance.
CsA treatment, 3,000 islets transplanted intraportally (blue), controls - no CsA, 2,000 islets (white), 2,500 islets (pink), 3,000 islets (red).

The metabolic results of Group II (intra-portal islet allografts with temporary CsA) are summarized below, defining basal, peak and ratio peak-to-basal (stimulation index, SI) response, in addition to the glucose decay constant (KG). Overall differences in basal graft insulin output were statistically significant (p<0.001, Kruskal-Wallis ANOVA), with values at two days and one day prior to hyperglycemia being significant by comparative testing ($p<0.05$, Dunn's method). The steepest fall in basal insulin output occurred on the day of hyperglycemia (27.5±5.5 vs. 5.8±0.9µU/ml, $p<0.01$, see figure 5.9 below).

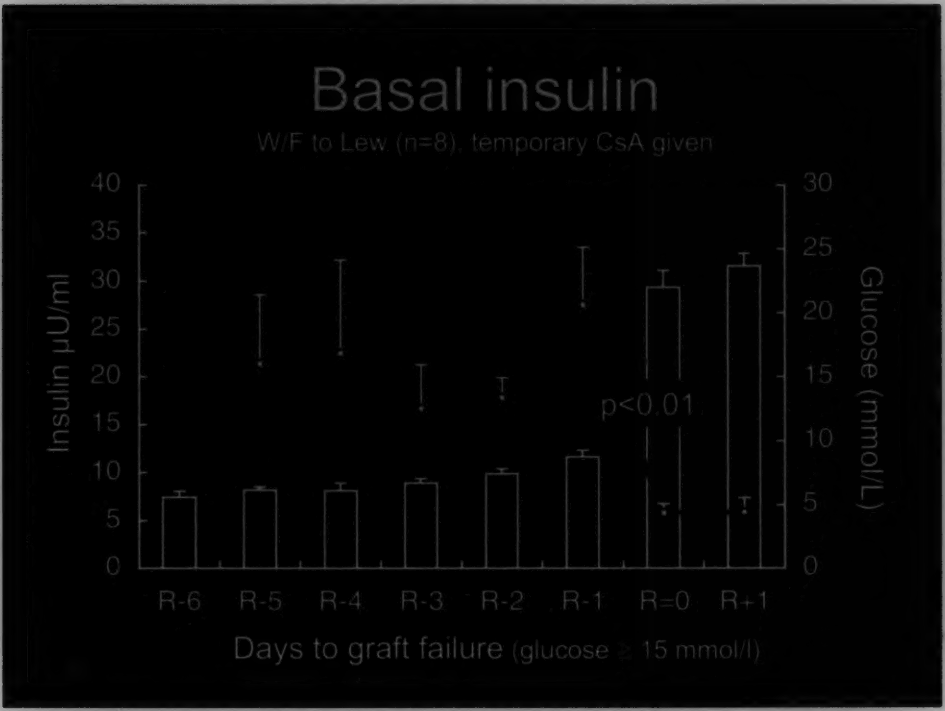


Figure 5.9: Decay in basal insulin output versus days to onset of graft rejection (defined by serum glucose > 15mmol/L).
Insulin (green) and glucose (blue)

Overall differences in phase I stimulated insulin levels (2min post iv glucose) were also highly significant ($p<0.001$, ANOVA), with output at two days prior to hyperglycemia reaching significance ($p<0.05$, see figure 5.10 below). Again, the most dramatic fall in peak insulin output occurred in synchrony with hyperglycemia. Differences in overall insulin stimulation index ($p<0.001$) paralleled changes seen in basal and peak insulin output (see figure 5.11 below).

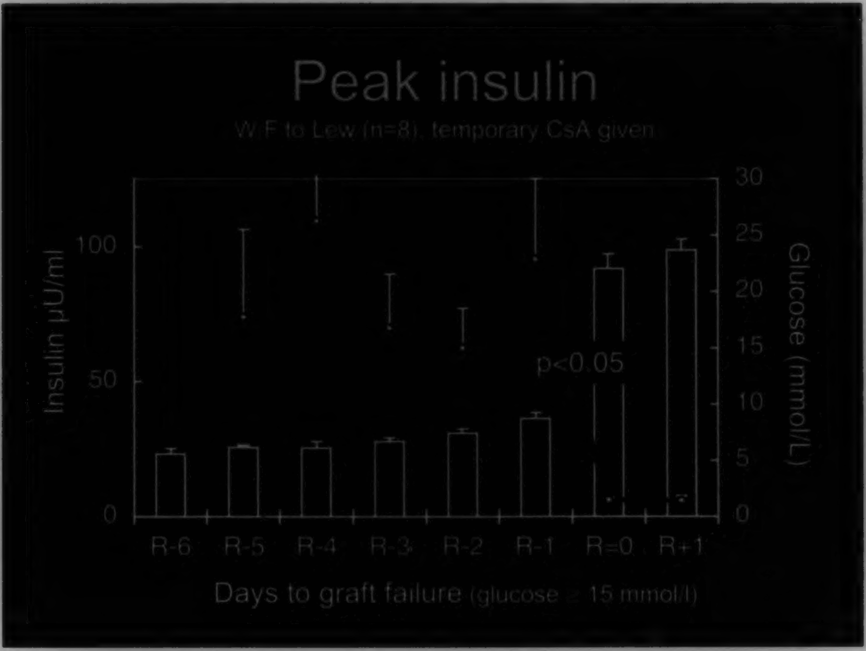


Figure 5.10: Decay in peak insulin output versus days to onset of graft rejection (defined by serum glucose > 15mmol/L).
Insulin (red) and glucose (blue)

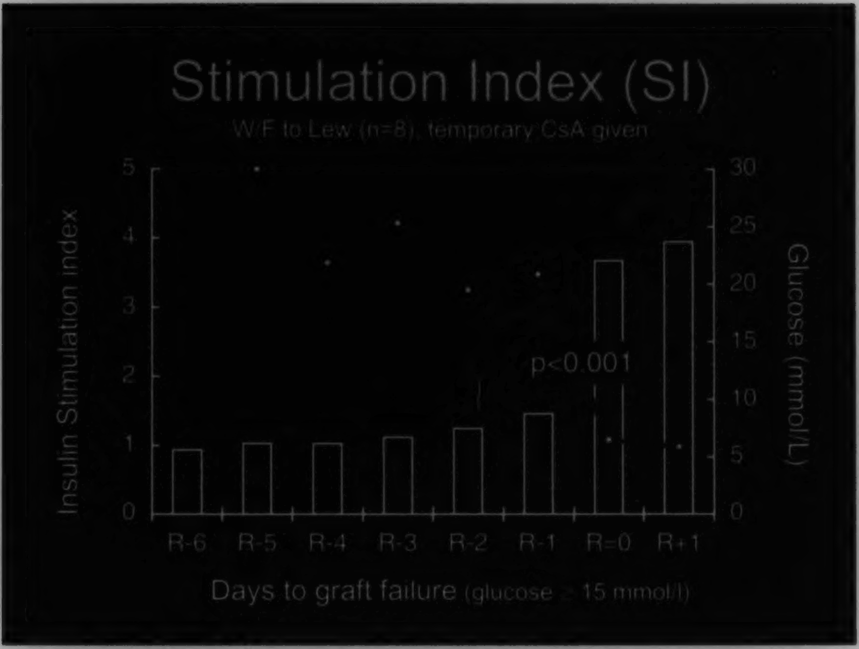


Figure 5.11: Decay in insulin stimulation index (SI) versus days to onset of graft rejection (defined by serum glucose > 15mmol/L).
Insulin stimulation index (red) and glucose (blue)

The most dramatic rejection-mediated change in graft metabolism were shown by serial decline in the glucose decay constant (K_G), with overall differences found to be highly significant ($p<0.001$, by ANOVA) (see Figure 5.12 below). The fall in K_G was most significant between the third and second days prior to hyperglycemia (2.58±0.3 vs. 1.63±0.2 %/min respectively, $p<0.001$).

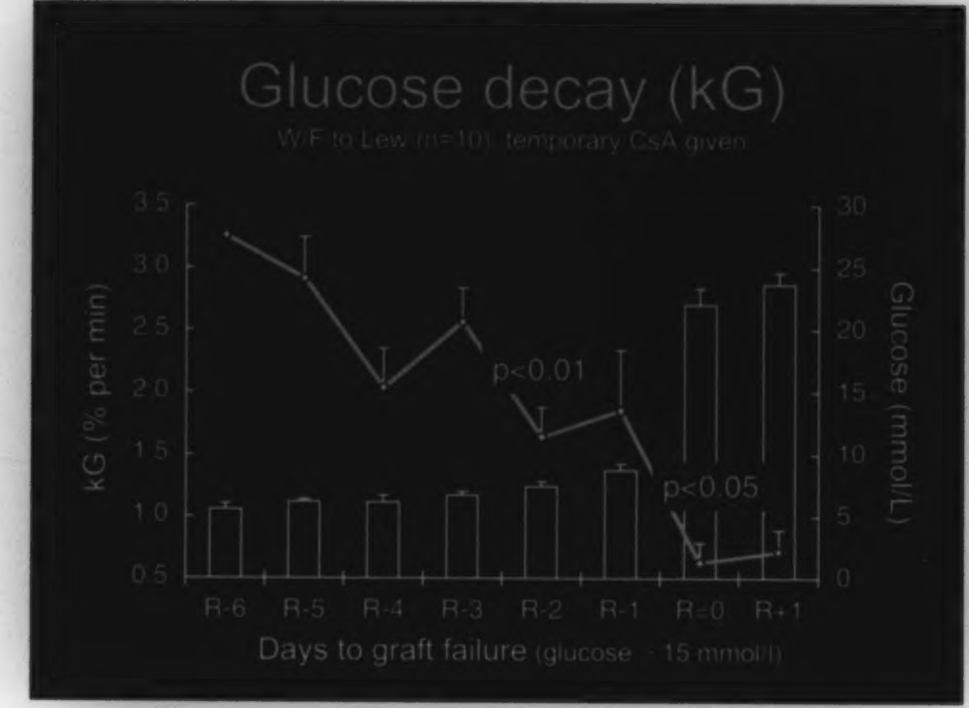
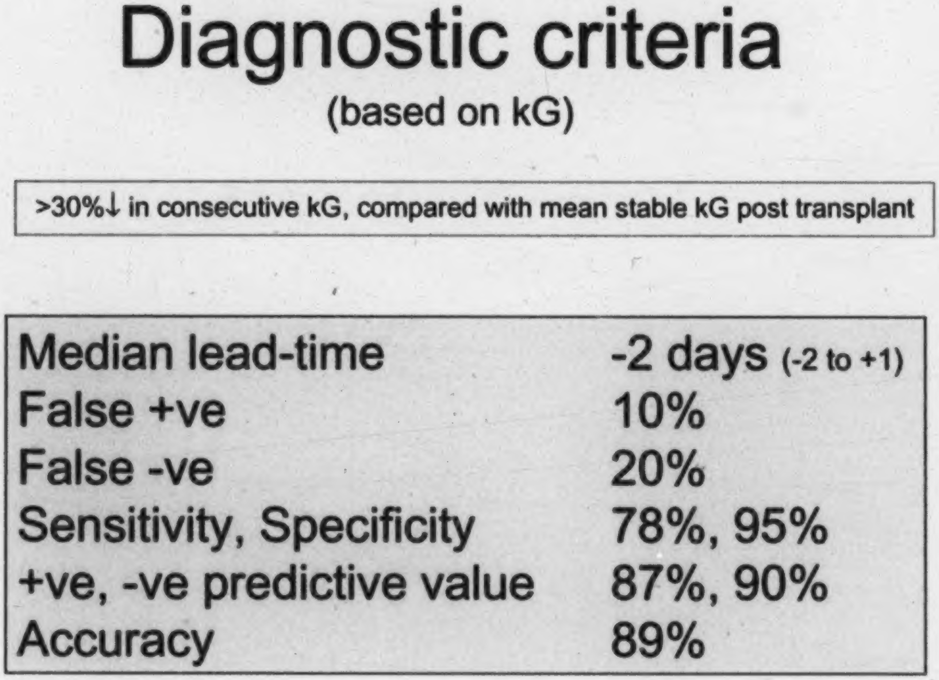


Figure 5.12: Decay in glucose decay constant (kG) versus days to onset of graft rejection (defined by serum glucose > 15mmol/L), calculated from an abbreviated 30 min glucose tolerance test.
KG (red) and glucose (blue)

Based on the above findings of sequential deterioration in K_G, we defined set criteria for diagnosis of early islet rejection as >30% decline in consecutive K_G compared to stable baseline values early after transplant. Applying the criteria to the sequential data above, the K_G test diagnosed early islet rejection with a median lead-time of 2 days ahead of hyperglycemia, with an accuracy of 89% and high sensitivity.



Diagnostic criteria

(based on kG)

>30%↓ in consecutive kG, compared with mean stable kG post transplant

Median lead-time	-2 days (-2 to +1)
False +ve	10%
False -ve	20%
Sensitivity, Specificity	78%, 95%
+ve, -ve predictive value	87%, 90%
Accuracy	89%

Table 5.3: Diagnostic criteria for early diagnosis of islet allograft rejection, based on a 30% decline in consecutive kG, compared with baseline

5.5 DISCUSSION

An inability to diagnose and treat early rejection of clinical islet allografts was a major component contributing to an 8% insulin-independence rate seen previously in clinical islet transplantation worldwide(*38*, *39*). Without concurrent functional monitoring, and assuming that untreated severe rejection culminates in eventual graft loss, the extrapolated one-year survival of liver, kidney and pancreas allografts with CsA/glucocorticoid-based therapy would likely have been of the order of 40-60%, rather than in excess of 90% enjoyed by most centers currently(*1*).

The major advance in recent outcomes in clinical islet transplantation with complete control of both acute rejection and autoimmune recurrence in a glucocorticoid-free regimen used in the "Edmonton Protocol" has lessened the need for early diagnostic markers of islet rejection(*5*). The further development of tolerance-inducing strategies shortly to be evaluated in clinical transplantation may one day relinquish the need for immune and functional monitoring completely, but at the present time careful graft surveillance, biopsy and appropriately directed intensification of therapy form an integral part of post-transplant care. This is acutely relevant for clinical islet transplantation, as the drastic sequelae of excessive immunosuppression can rarely be justified for any non-life-saving therapy.

Development of diagnostic markers for allograft rejection has proven to be particularly challenging for an endocrine islet transplant. The engrafted cell mass is less than 0.1% of that of a liver transplant, and is therefore not readily amenable to percutaneous biopsy when embolized to the intrahepatic site (although this has been achieved on rare occasions)(*40*). Except for the new onset of hyperglycemia, which is an irreversible event in the experimental setting and impossible to gauge in recipients on insulin, there are no specific serological markers of islet rejection available to date.

Vascularized pancreas transplantation does not suffer from these limitations, in that normoglycemia with complete insulin-independence is readily achieved in virtually all recipients despite the use of diabetogenic immunosuppression, but this procedure is associated with increased morbidity. Recent trends in pancreas transplantation are shifting towards physiologic intra-portal insulin delivery with enteric exocrine drainage(*41*). Hypoamylasuria has not proven to be as specific a marker of pancreatic rejection as originally thought, and one recent study showed that only 45% of patients with falling urinary amylase actually had biopsy-proven rejection(*42*). The simultaneous transplantation of a kidney was originally regarded as an essential immunologic "barometer" for pancreatic rejection, with treatment of biopsy-proven renal rejection affording pancreatic protection. The combined use of tacrolimus, mycophenolate, and glucocorticoid with antibody induction has recently permitted equivalent successful outcome for solitary pancreas transplantation with enteric exocrine drainage(*43*). Careful serum monitoring of amylase and lipase, with liberal use of percutaneous ultrasound-guided pancreatic biopsy, has been an important component in this approach(*44, 45*). The transplanted vascularized pancreas clearly has more physiological reserve than isolated islets, and both in the experimental and clinical setting it has been possible to reverse severe pancreatic rejection to restore normoglycemia(*46*).

The current study explores three novel approaches towards early diagnosis of islet allograft rejection. A search for endogenous islet-specific candidate markers led to an initial study of GAD_{65} activity in a series of autografted and allografted dogs under temporary immunosuppression. GAD_{65} isoforms have been implications in the induction and accelerated destruction of b-cells in autoimmune diabetes in humans, and justified study as a serum marker of islet destruction in transplanted islets(*8, 9, 47*). While perturbations in serum GAD_{65} activity were detectable in allograft serum using a $^{14}CO_2$-

release assay, the effect was striking in only one of six animals studied. Although statistical significance was not attained, the study clearly demonstrates that an islet-specific protein may be detected serologically prior to hyperglycemia. This is an important finding, as it demonstrates that the kinetics of immune-mediated destruction of a cell-transplant preparation may exceed the capacity of local phagocytotic and antigen-breakdown mechanisms, leading to the release of free protein/enzyme into the serum. It is unclear why rejection-mediated elevations in serum GAD_{65} were not shown consistently in all animals, but this may reflect: a) enhanced macrophage degradation of free GAD_{65} in an intrasplenic site, b) variability in islet engraftment mass, coupled with c) inadequate sensitivity of the $^{14}CO_2$-release GAD_{65} assay. A further anticipated difficulty in the application of GAD_{65} antigen rejection monitoring in clinical islet transplantation is that antibodies to GAD_{65} persist in the serum of diabetics, although the absolute amount may decay with progression of disease(*48, 49*). These antibodies may potentially scavenge free GAD_{65} antigen before it reaches the limit for detection. Jaeger *et al* have shown that systemic immunosuppression in islet-kidney transplantation does not eliminate GAD_{65} antibodies, and have implicated these antibodies in the early autoimmune failure of islet grafts(*50*).

Frustrations with development of an endogenous islet-specific serological marker led to the concept of genetically engineering islets to express a unique exogenous marker to serve as a "sentinel signal" for early rejection. The concept was explored experimentally using the replication-deficient adenoviral vector Ad-5 CMV b-gal. There are several practical limitations of this approach if it were to be extended to the clinical setting, including: a) the limited duration of adenoviral expression, b) the theoretical risk of viral-mediated immunologic enhancement and c) the unknown potential for induction of malignancy in the immunosuppressed host. It has recently been shown by Murave *et* *al* that expression of adenoviral gene products may persist as long as 20 weeks after transplantation(*15*), encouraging the applicability of this technique for early immune monitoring. Several studies have applied various adenoviral gene therapies in an attempt to prolong islet allograft or xenograft survival, but the overall results have been disappointing thus far. While Deng *et al* were able to prolong canine islet xenograft survival and reduce primary non-function with immunosuppressive cytokines(*18*), strategies enhancing Fas-ligand or IL-4/IL-10 expression failed to prolong graft survival(*32, 51*).

X-gal staining of transduced and control islet preparations revealed dense b-gal activity *in vitro*, confirming satisfactory gene transfer. Dynamic perifusion of transduced and control islets in low and high glucose media showed that normal *in vitro* islet insulin secretory capacity was maintained despite viral manipulation, thus confirming the earlier findings of Csete *et al*(*14*). Intravenous injection of healthy rats with pure b-gal enzyme confirmed stability of the unique viral product in serum, and indicated that the dynamics of b-gal clearance conformed to a stable log-half-life, reaching background levels within 8 hours – characteristics that seemed ideal for serum "sentinel signal" monitoring. The rodent Ad-5 CMV b-gal allograft transplants rejected their grafts with a similar tempo to non-transduced allografts, suggesting that viral manipulation did not alter graft immunogenicity. These experiments revealed that exogenous b-gal could be detected readily in serum with bimodal release during acute rejection. The fact that serum b-gal levels were twenty times higher than baseline during the islet engraftment phase after transplantation may have masked earlier less marked elevations, limiting the effectiveness of this exogenous marker for diagnosis of early islet rejection. It is important to note that the relatively high islet engraftment mass used in this study (3,000 islets delivered intraportally) corresponds approximately to the mean threshold mass of

11,000 IE/kg found clinically to facilitate insulin independence(5). A lower islet engraftment mass would likely have also been detectable by serum monitoring, but was not used for these studies since a lower engraftment mass was less effective at providing routine reversal of hyperglycemia in preliminary studies. The chemilumescent super-sensitive assay was readily able to detect peak concentrations of 2.0 x 10^{-3} iU b-gal per cc in serum. Having established the concept of "sentinel signaling" in diagnosis of islet rejection, future advances in gene therapy with use of lipofection, biolistic transformation with the "gene gun" or transgenic strategies may allow further exploration of this approach.

Development of an abbreviated glucose tolerance test performed over just 30 minutes on a daily basis offered the greatest promise as an early diagnostic marker of islet rejection in the current study. We found that basal and peak graft insulin output fell significantly on the days preceding hyperglycemia, but was elevated immediately preceding graft failure. This interesting finding has been described previously by Jaeck *et al*(*52*), and presumably reflects insulin release from disrupted or stressed b-cells. Insulin and c-peptide monitoring have been used to monitor clinical islet graft function, but have not shown utility for early diagnosis of islet rejection – one limiting factor being that the detailed assay techniques make timely acquisition of data a challenge. The most striking decline was seen in K_G, with a 30% fall from baseline permitting diagnosis of early islet rejection with high specificity, sensitivity and accuracy, and with a two day lead time ahead of elevation in fasting blood sugar. If these results can be confirmed in large animal pre-clinical models and ultimately in clinical islet transplant recipients, the simplicity of a four blood-drop, 30 minute test with K_G determination could be readily adapted for early islet rejection monitoring.

Glucose tolerance tests have been used to define a pre-diabetic state, and several investigators have shown blunting of first phase insulin secretion in islet cell antibody positive siblings of type 1 diabetic patients(*53, 54*). Serial standard (non-abbreviated) glucose tolerance tests have been studied previously to diagnose early rejection in pancreas transplantation, but not in islet transplantation to our knowledge. Bewick *et al* performed heterotopic vascularized pancreas transplants in dogs, and found deterioration in K_G up to 3 days before elevation in fasting blood glucose in immunosuppressed recipients(*55*). There was also a rise in plasma insulin level occurring at 24 to 48 hours before the rise in fasting blood sugar in their study. Henry *et al* compared hypoamylasuria with glucagon and glucose-stimulated IVGT tests in type 1 diabetic kidney-pancreas recipients, and found that a blunted first-phase insulin response was a more specific indicator of impending b-cell failure(*56*). Elmer and colleagues reported that K_G was as sensitive as urinary amylase or serum anodal trypsinogen for diagnosis of pancreas rejection in their hands(*37*). Elmer *et al* further showed that a >20% decline in K_G was associated with a 72% incidence of acute biopsy-proven or treatment-responsive pancreatic rejection, and that the test had a sensitivity of 89%, specificity of 91% and overall accuracy of over 90%(*57*). Perturbations in K_G due to insulin resistance from increase in diabetogenic pharmacotherapy occurred in 19% of cases. High dose corticosteroid treatment, but not low dose, has been shown to impair K_G(*58*), as has major sepsis(*59*), prolonged anesthesia and surgery(*60*), increased body mass index and renal failure(*61*), representing limitations to the interpretation of the K_G test in some patients.

In conclusion, the current study has explored three new approaches for early diagnosis of islet allograft rejection utilizing endogenous GAD65, exogenous b-gal adenoviral expression and by abbreviated serial glucose tolerance testing. GAD_{65} monitoring was not effective, possibly as a result of assay insensitivity or due to rapid scavenging of free GAD_{65} in an intrasplenic site. Sentinel signal serum monitoring of b-gal did show marked elevation during islet rejection, but there was no lead-time over onset of hyperglycemia detectable in our hands. The most promising marker of islet allograft rejection proved to be serial abbreviated glucose tolerance testing, which gave a two-day lead-time over elevation in fasting blood sugar. The lead-time of two days may seem relatively short, but it should be appreciated that the acute unmodified rejection model used in the above experiments followed acute and complete withdrawal of all immunosuppression – a situation that would arise only rarely in clinical practice. In most instances acute rejection follows sub-therapeutic immunosuppression rather than sudden and complete withdrawal, and in this situation the aggressiveness of the response is more subdued. Therefore the potential for early intervention to reverse early islet rejection would be greater in this setting. Thus, once stable graft function has been achieved and the rejection tempo has been slowed by chronic maintenance therapy, serial glucose tolerance tests might only be required on a much less frequent basis such as one weekly rather than on a daily basis. The potential for patient self home-testing of four time-point glucose meter readings after standard oral glucose load may make this an effective and practical strategy until more specific immunologic markers can be developed for clinical islet transplantation – comparable to the urine cytology PCR-based tests that are proving useful currently in renal transplant rejection(*62, 63*). Roep et al have recently shown that peripheral blood T cell reactivities were perturbed, with increases in precursor frequencies of graft-specific alloreactive T-cells following acute rejection, suggesting that peripheral T cell events might serve as an alternative potential marker of early islet allograft rejection(*64*).

Indeed, as new tolerance induction strategies are developed for clinical application, they may be evaluated initially under the cover of sirolimus maintenance monotherapy, based on its tolerance-permissive effects on apoptotic cell death of activated lymphocytes (*65*). This would further act to modify the aggressiveness of any acute rejection response, favoring the potential utility of acute rejection monitoring by serial glucose decay. Further studies are clearly required to determine whether acute intervention during early islet allograft rejection could lead to graft rescue.

5.6 ACKNOWLEDGEMENTS

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CHAPTER 5 (APPENDIX):

HIGH YIELD OF RODENT ISLETS WITH INTRADUCTAL COLLAGENASE AND STATIONARY DIGESTION - A COMPARISON WITH STANDARD TECHNIQUE

NOTE: A previous version of this appendix was published in Cell Transplantation 1996: vol 5 (6), 631-638
Authors on this paper included:
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5A.1 ABSTRACT

Intraductal distention of the pancreas with collagenase followed by stationary warm incubation improves the recovery of islets of Langerhans in the rat, but controlled studies are needed for valid comparison with standard isolation methods. We have modified Gotoh's technique of stationary digestion for high yield isolation in the rat (*Stationary*). The method is subjected herein to rigorous blinded comparison with the standard chopped tissue (*Chopped*) technique, based on Lacy et al, as performed in our laboratory for over 10 years. Islet recovery was determined by a single observer 'blinded' to the method of isolation used, and only intact islets of diameter ≥ 100 μm were included. *Stationary* gave 719 ± 114 islets per pancreas (mean ± sd, n=21 isolations) vs 487.5 ± 69 for *Chopped* (n= 36 isolations), a 47.5% increment in yield ($p < 0.0001$). In vitro islet perifusion showed no statistical difference in stimulation index (SI) or stimulated area under the curve (SAUC) between the two methods, but *Stationary* showed a trend towards improved phase II insulin release. In vivo function was assessed by isogeneic transplantation of 2,000 islets beneath the renal capsule of streptozotocin diabetic recipients (65 mg/kg Sigma); *Stationary* recipients (n=7) became normoglycemic (≤ 8 mmol/L) by 3.3 ± 4.8 days vs 1.6 ± 1.5 days for *Chopped* recipients ($p = 0.4$ ns, mean ± SEM). IVGTT performed at 1 month posttransplant gave K-values for *Stationary* of 2.64 ± 0.8 vs 2.62 ± 0.8 for *Chopped* (mean ± sd, p=0.9 ns, n=6, unpaired T-test), which were not distinguishable from normal control rats (2.59 ± 0.8) ($p = 0.9$ ns, n=10). Graft function remained stable until graft bearing nephrectomy induced hyperglycemia uniformly within one day. Graft histology showed healthy well preserved structure on light microscopy, with well granulated beta cells on EM. Economic costs of rat, collagenase and Ficoll were 26% ($50.82) lower per recipient for *Stationary*. We

conclude that modified stationary digestion significantly improves islet recovery with excellent in vitro and in vivo function, and is cost effective.

5A.2 INTRODUCTION

The standard chopped tissue digestion (*Chopped*) process in current use in several islet laboratories was introduced by Moskalewski in 1965(1) and substantially modified by Lacy and Kostianovsky for rodent islet isolation(2). The method involves acinar disruption by pancreatic insufflation of a salt solution via the pancreatic duct, chopping the gland into tissue fragments, and subsequent incubation and agitation of the preparation in collagenase enzymes at 37∞C. The degree of tissue mincing, vigor of agitation and the timing of digestion end-point are all critical factors in the prevention of islet damage or loss, making the process operator dependent. Batch-to-batch variation in collagenase activity further compounds inconsistency in islet recovery. It has been estimated that at best only 50% of the islets contained within the donor rodent pancreas are preserved during isolation(3). Thus, for rapid reversal of the streptozotocin-induced diabetic state in the rat (2,000 islets) using standard isolation techniques, between 4 and 6 donor pancreata are required.

Intraductal distention of the pancreas with collagenase solution followed by a period of warm incubation is the established method for optimal islet recovery from the dog(4,5), pig(6), and human(7,8) gland. An adaptation of this method, with stationary incubation for more gentle dissociation, improves recovery of mouse(9) and rat islets(10,11). Stationary digestion is attractive because it eliminates a series of critical operator dependent variables, and may thus allow for improved consistency in yield. Controlled studies comparing stationary digestion to standard techniques in the rat are needed to conclusively establish benefit before the method is adopted universally by islet laboratories.

We have developed a modified *Stationary* digestion technique which permits reliable isolation of large numbers of rodent islets. The aim of this study was to subject the modified *Stationary* method to a rigorous blinded comparison against the standard *Chopped* technique as carried out in our laboratory for over 10 years.

5A.3 MATERIALS AND METHODS

5A.3.1 Experimental design.

A blinded comparative randomized assessment of *Chopped* vs *Stationary* isolation techniques was carried out in a single laboratory, controlling for donor rat weight, strain, identical collagenase batch, counting methodology and transplantation technique.

The following outcome parameters were compared: islet recovery, in vitro insulin release in response to dynamic glucose challenge on perifusion, and function after isogeneic transplantation. *Chopped* and *Stationary* islet recipients were assessed for time to normoglycemia posttransplant, and IVGTTs were carried out at 1 month (0.5 g/kg glucose IV) to determine K-values compared with normal non-diabetic controls.

Animals and the induction of diabetes. Male inbred Wistar-Furth (WF) rats ($RT1^U$) of weight range 250 - 275g were obtained from Harlan Sprague-Dawley inc., Indianapolis, USA and used as islet donors and recipients. Diabetes was induced by a single intravenous injection of streptozotocin (65 mg/kg body weight) via the penile vein

(Sigma lot 83H0014, St. Louis, MO, USA). Rats were considered diabetic after non-fasting glucose levels were confirmed ≥ 18 mmol/L on 3 or more occasions over a period of at least 7 days prior to transplantation. Glucose monitoring was performed on capillary tail vein whole blood samples using a portable blood glucose sensor (Companion II" meter and test strips, Medisense Canada Inc.) Rat husbandry was carried out in accordance with the standards outlined by the Canadian Council on Animal Care.

Anesthesia and surgery. Donor rats were anesthetized with a single intraperitoneal injection of sodium pentobarbital (0.4 mg/g body weight). After midline incision, mosquito clamps were applied to the distal bile duct at its point of entry into the duodenum, and proximally to above the bifurcation of right and left hepatic ducts. The bile duct was incised with micro-iris scissors and canulated below the bifurcation with PE50 tubing attached to a 26-gauge needle and syringe(2). The tubing was secured in place with a 4/0 silk ligature, care being taken to prevent the tip of the tubing from migrating distally beyond 2 - 3 mm to allow adequate distention of the pancreatic head.

Standard chopped tissue digestion method. Standard *Chopped* isolations were carried out after Lacy and Kostianovsky(2) by retrograde pancreatic distention with 10 mls of cold supplemented Hank's balanced salt solution (HBSS, Gibco, Grand Island NY) containing 100mg/dl D-glucose (Abbott Laboratories Ltd., Montreal PQ), 100 U/ml penicillin (Whittaker Bioproducts, Walkersville MD), and 100 µg/ml streptomycin (Whittaker Bioproducts). After pancreatectomy and lymphadenectomy the tissue was minced into 1-2 mm fragments using curved scissors, digested in collagenase (type V, Sigma lot 10H6828) at a concentration of 7.5 mg/ml (15mg per g pancreas) in supplemented HBSS (1 pancreas per 50 ml Falcon tube) and manually agitated for 11 minutes at 37∞C. Digestion was stopped by addition of 30 mls of cold supplemented

HBSS followed by centrifugation (600 G), then 3 serial rinse cycles prior to resuspension in 10 ml of 25% Ficoll. Purification was on discontinuous gradients (25%, 23%, 20.5% and 11%) of Sigma Ficoll 400-DL made up in HBSS containing HEPES (12.5 ml HEPES : 500 ml HBSS) (N-2-Hydroxyethyl-piperazine-N'-2-ethane-sulphonic acid) (Gibco)(12). Islets were recovered from the upper two interfaces after 10 minutes centrifugation (800 G, no break), washed in Medium 199 (Gibco) supplemented with 10% new-born calf serum (Gibco), penicillin (100 U/ml) and streptomycin (100 μg/ml) .

Modified stationary digestion method. After ductal canulation pancreata were distended maximally with 20 ml collagenase (1mg/ml Sigma type V 10H6828) using a pulsed infusion technique. Pancreata were retrieved and stored on ice for 30 minutes in individual T-flasks (Falcon 50 ml tissue culture flasks, Becton Dickinson) prior to stationary digestion at 37∞C for 35 minutes. The preparation was then transferred to 50 ml tubes (Falcon, Becton Dickinson), made up to a volume of 20 mls with HBSS (containing PSD), and vortexed as above to dissociate the islets from adherent acinar elements. The suspension was centrifuged (1,500 rpm (600G) for 5 seconds), residual fat was aspirated, and the pellet washed and spun twice in supplemented HBSS (1,000 rpm (400G) for 5 seconds) prior to passage through a mesh filter (pore size 850μm). Any residual tissue fragments were broken down by spraying with HBSS from an 18G needle and 20 ml syringe. The tissue was then spun at 1,500 rpm (600G) for 5 seconds, and purified on Ficoll gradients (as above, but spun for 15 minutes at 2,000 rpm, 800G). Islets were retrieved from the upper two interfaces, resuspended in supplemented Medium 199, and pooled into one 50 ml tube. The islet preparation was then exposed briefly to Ficoll again (5 minutes at 800G) to improve islet purity. Islets were recovered from the upper two interfaces, then washed three times in M199 prior to counting.

Assessment of yield. Islet yield was assessed by a single observer 'blinded' to the method of isolation used, and only intact islets of diameter ≥ 100 μm were included. Islet identification was aided by the use of a binocular dissecting microscope (x 25) with background green illumination with white side-lighting(13). Islets were sized using an ocular grid (Wild Leitz, Willowdale), and were collected for viability and functional evaluation.

Viability assessment in vitro. The perifusion technique of Lacy et al(14) was used for dynamic in vitro glucose challenge to evaluate insulin release from islets (200 per chamber) after overnight culture in standard RPMI 1640. 200 islet aliquots were transferred to small chambers and perifused with RPMI1640 (Gibco) containing 2.8 mmol/L glucose for one hour initially followed by 28 mmol/L glucose for one hour, and subsequent 2.8 mmol/L for the final hour. The effluent from the perifusion chamber was sampled twice in low glucose environment (51 and 59 minutes), at 4 time points during exposure to high glucose (65, 69, 89 and 109 minutes) and again in low glucose (149 and 179 minutes). Insulin content was determined using insulin double antibody radioimmunoassay kits (Pharmacia Diagnostics, Uppsala, Sweden) and rat insulin standards (Novo Research Institute, Bagsvaerd, Denmark)(15). The stimulation index (SI) (mean insulin release during high glucose exposure over the mean of the pre and post challenge low glucose baselines)(16) and stimulated area under the curve (SAUC) were then calculated(17).

Isogeneic transplantation. 2,000 islets were transplanted beneath the renal capsule of male streptozotocin diabetic W/F rats (weight 250-275g). Non-fasting (09.00h) capillary tail vein glucose was tested daily for two weeks, and 3 times per week thereafter until the termination of the experiment.

Functional assessment in vivo. Intravenous glucose tolerance tests (IVGTT), expressed as a K-value(18), were performed at one month posttransplant. Results in both groups were compared with control K-values in normal (non-diabetic) rats of the same strain. Femoral venous and arterial canulae were placed under general anesthesia for bolus glucose delivery (0.5g/kg 50% dextrose) and sampling respectively. On completion of the glucose challenge the graft-bearing kidney was excised for histology, and the rat kept alive for a further 24 hours to exclude spontaneous remission of the streptozotocin diabetic state.

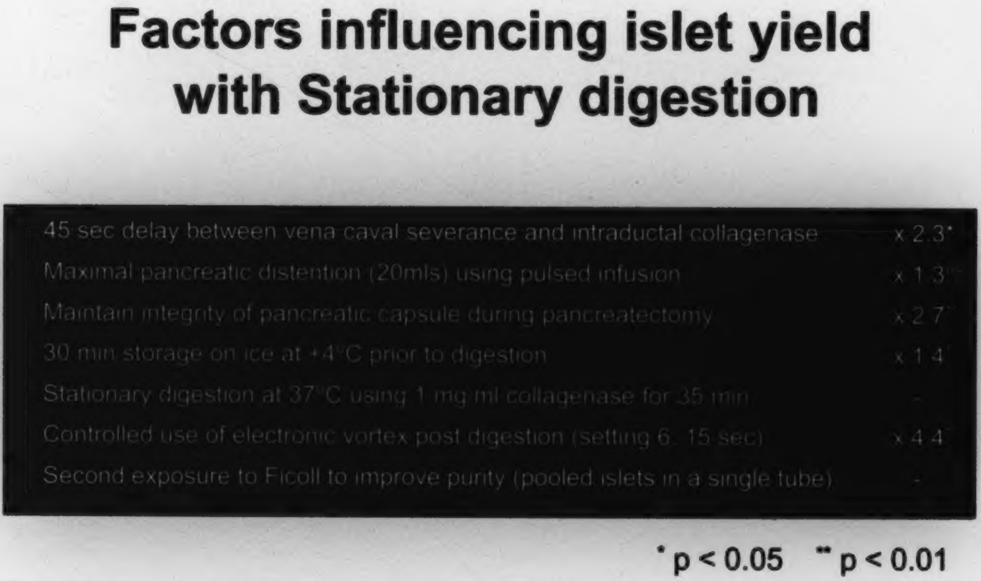
Statistical analysis. Islet yield per pancreas was expressed as the mean ± sd, and compared using an unpaired T-test (2-tailed, heteroskedastic variance). Similar tests were used to compare stimulation indices (SI) on perifusion, time to normoglycemia posttransplant, and K-values from IVGTT at one month. In all cases statistical difference was assumed if the probability (p) was < 0.05.

5A.4 RESULTS

5A.4.1 DEVELOPMENT OF MODIFIED STATIONARY DIGESTION.

Preliminary dose-time response studies were performed with collagenase type V (Sigma 10H6828) using the original stationary digestion method as described by Gotoh et al(5). Optimal stationary digestion was achieved with a collagenase concentration of 1 mg/ml and an incubation period of 35 min at 37∞C. We found variability in yield from isolation to isolation using Gotoh's original technique, and our islet preparations were often contaminated by exocrine debris. After a critical appraisal of factors affecting yield, we have defined modifications to Gotoh's technique which we found to be essential for improved consistency and maximal islet yield in our hands (see Table 5(A).1 below):

i) Effect of intact circulation. Early on we were troubled by the rapid onset of hemorrhagic pancreatitis occurring predominantly in the tail of the gland after collagenase injection, and under these circumstances it was rarely possible to isolate adequate numbers of islets. The problem was resolved by ensuring that the systemic circulation was interrupted with vena caval and aortic transection for a period of 45 seconds prior to pancreatic distention with collagenase.



Factors influencing islet yield with Stationary digestion

45 sec delay between vena caval severance and intraductal collagenase	x 2.3*
Maximal pancreatic distention (20mls) using pulsed infusion	x 1.3**
Maintain integrity of pancreatic capsule during pancreatectomy	x 2.7**
30 min storage on ice at +4°C prior to digestion	x 1.4*
Stationary digestion at 37°C using 1 mg ml collagenase for 35 min	-
Controlled use of electronic vortex post digestion (setting 6, 15 sec)	x 4.4**
Second exposure to Ficoll to improve purity (pooled islets in a single tube)	-

* $p < 0.05$ ** $p < 0.01$

Table 5(A).1:Critical factors influencing islet recovery in development of the stationary digestion technique

ii) *Achieving maximal pancreatic distention.* Islet recovery decreased by a factor of 2.7 if the integrity of the pancreatic capsule was disrupted during distention

($p<0.01$). Controlled techniques were developed therefore to improve the consistency and degree of pancreatic distention. When pulsed injection was used, relinquishing pressure intermittently to prevent leakage from the pancreatic capsule, the pancreas could be distended reliably with a volume of 20 mls collagenase solution. Elevation of the spleen during distention helped to ensure optimal distention of the pancreatic tail. Islet recovery was improved by a factor of 1.3 (ns) using pulsed distention.

iii) *30 minute hold period at +4∞C.* It was found that islet yield improved by a factor of 1.4 ($p<0.05$) after storage of pancreata on ice for 30 minutes in T-flasks prior to stationary digestion.

iv) *Controlled vortex.* Controlled dissociation of the post-digested tissue on an electronic vortex (Vortex Genie, Scientific Industries Inc., NY), filling tubes to exactly 20 ml each time (setting 6, 15 seconds), improved consistent liberation of undamaged islets by a factor of 4.4 ($p<0.01$).

5A.4.2 COMPARISON OF STATIONARY VS CHOPPED METHODS.

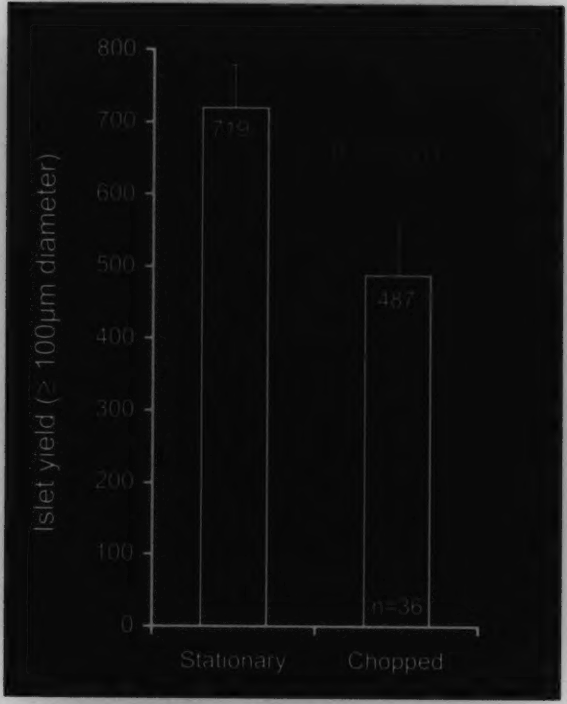


Figure 5(A).1: Islet yield from modified stationary digestion (*Stationary*) vs control chopped tissue digestion (*Chopped*)

Islet yield. Islet recovery was 47.5% higher with the stationary method (Refer to Figure 5(A).1 above:.

In vitro function. The glucose stimulated insulin response of both islet groups elicited a biphasic pattern of insulin release after overnight culture, with a trend towards improved phase II insulin release in the *Stationary* group (ns) (see figure 5(A).3 below).

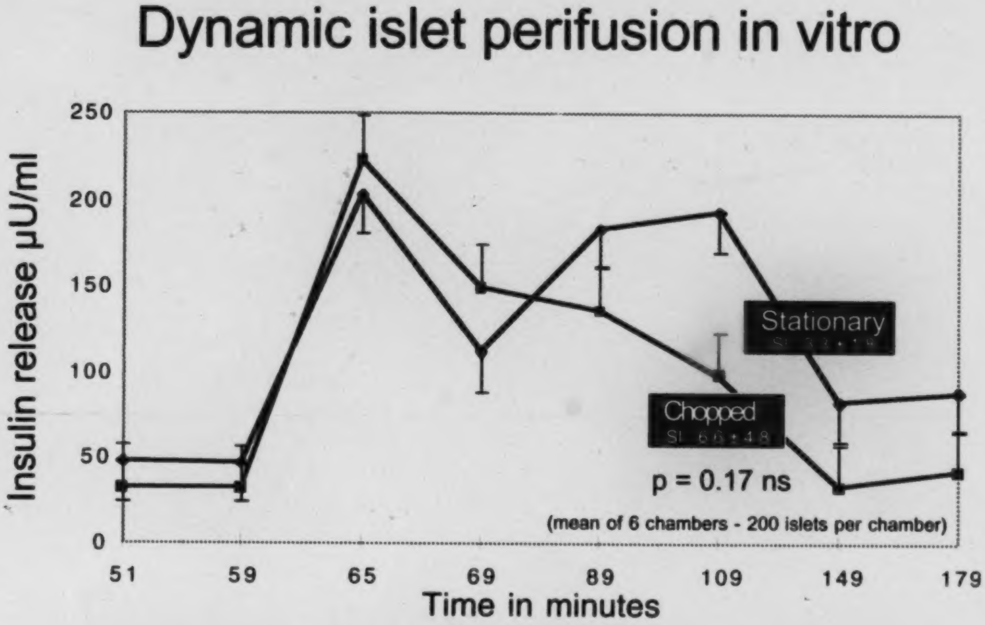


Figure 5(A).3: *In vitro* islet perifusion (*Stationary* vs *Chopped*) in dynamic glucose challenge, showing biphasic insulin release in both groups. The results are also expressed as stimulation indices (SI).

The post-hyperglycemic insulin release from *Stationary* islets failed to return to the pre-challenge baseline, and may reflect warm ischemic injury due to the prolonged digestion time (35 minutes) for this group. Stimulation indices for *Stationary* derived islets (3.3 ± 1.8) vs *Chopped* islets (6.6 ± 4.8) did not reach statistical significance (p=0.17). The SAUC for *Stationary* vs *Chopped* were $6.9 \pm 2.0 \times 10^3$ vs $6.0 \pm 1.2 \times 10^3$ respectively (p=0.7 ns).

Function in vivo. Transplantation of 2,000 isogeneic islets (≤100µm diameter) beneath the left renal capsule universally corrected the diabetic state (n=7 per group). Early graft function was expressed as the mean (± SEM) time to restore normoglycemia

(≤8 mmol/L) post-transplant; *Stationary* islet recipients took 3.3 (±4.8) (median 2.0, range 1-14) days vs 1.6 (± 1.5) (median 1.0, range 1-5) days for *Chopped* recipients (p=0.4 ns) (see figure 5(A).4 below):

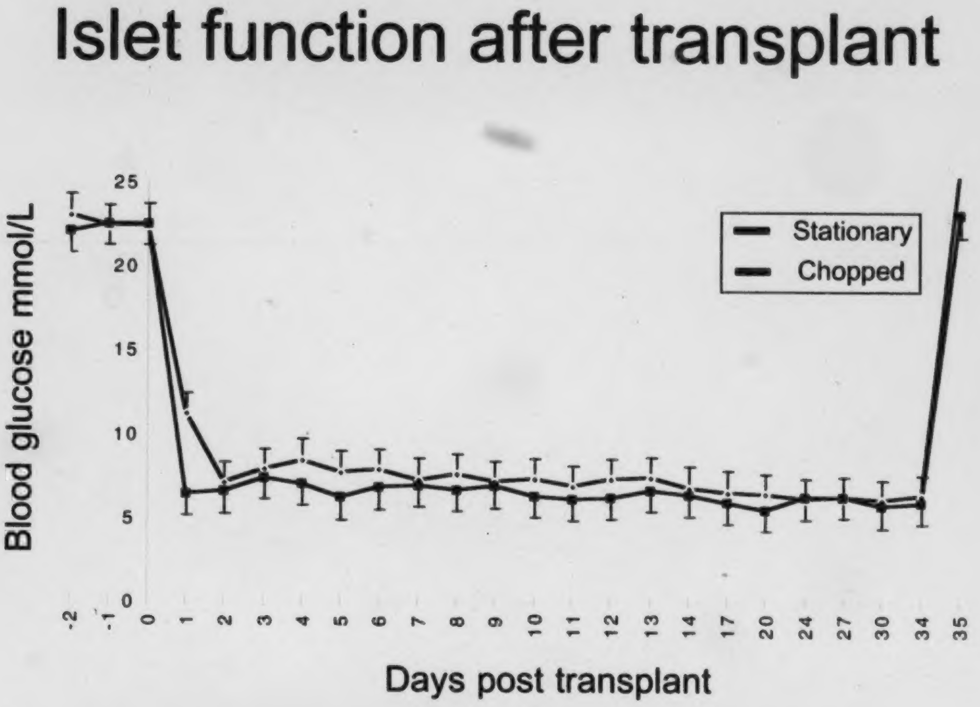
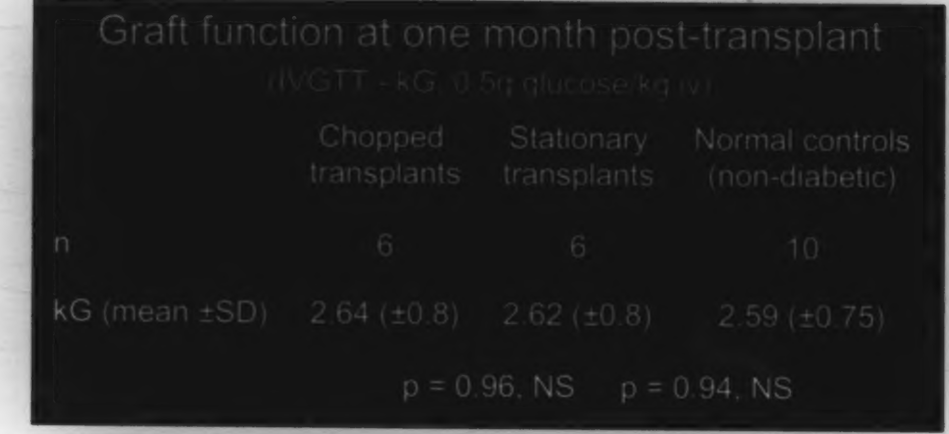


Figure 5(A).4: Graft function after transplantation of 2,000 islets placed beneath the renal capsule of isogeneic Wistar-Furth diabetic rats (mean ± SD, n=7 per group).

Excellent graft function was maintained for > 30 days, until graft-bearing nephrectomy induced hyperglycemia in all recipients within 24 hours.

Intravenous glucose tolerance (IVGTT) at one month after transplantation in both *Stationary* and *Chopped* recipients were indistinguishable from untreated normal control rats, and were indicative of excellent graft function in all groups (table 5(A).2 below).



Graft function at one month post-transplant

(IVGTT - kG: 0.5g glucose/kg iv)

	Chopped transplants	Stationary transplants	Normal controls (non-diabetic)
n	6	6	10
kG (mean ±SD)	2.64 (±0.8)	2.62 (±0.8)	2.59 (±0.75)
	p = 0.96, NS	p = 0.94, NS	

Table 5(A).2: Graft function at 1 month post-transplant, as assessed by kG on intravenous glucose tolerance testing of *Chopped* and *Stationary* recipients compared with normal non-diabetic controls.

Graft histology. Graft-bearing nephrectomy specimens were processed for immunohistochemistry (hematoxylin and eosin, and immunoreactive insulin) and electronmicroscopy (EM). *Stationary* recipient islet grafts showed good preservation of structure on light microscopy, and had well granulated beta cells on EM at one month (figure 5(A).5:). No difference in islet ultrastructure was evident between the two isolation methods.

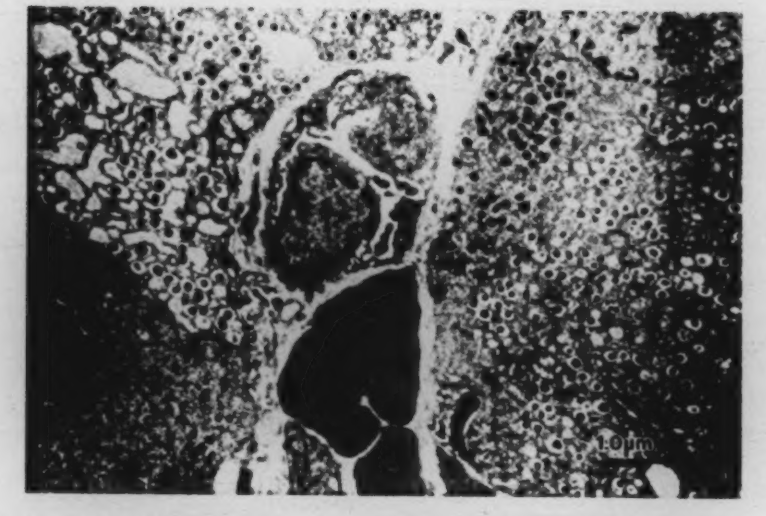


Figure 5(A).5: Electronmicroscopy of *Stationary* islet isograft beneath the renal capsule, with normal intracellular structure and insulin granules in the beta cell cytoplasm.

Economic analysis. Mean number of donor rats required to isolate 2,000 islets were 2.8 for *Stationary* vs 4.2 for *Chopped* (67% reduction in rats). Mean collagenase requirements per donor pancreas were 20.0 (± 0.1) mg for *Stationary* vs 24.9 (± 2.8) mg for *Chopped*, representing a 54% reduction in collagenase (n=18 isolations, $p < 0.0001$). Ficoll requirements per 2,000 islet isolation were 16.6g for *Stationary* and 18.3g for *Chopped*. The overall actual dollar costs (Canadian) per transplant recipient were $143.15 for *Stationary* and $193.97 for *Chopped* (a $50.82, 26% saving).

5A.5 DISCUSSION

Ductal injection of collagenase results in acinar disruption and breakdown of the islet-acinar interface, and a period of stationary warm incubation is claimed to improve islet recovery in the mouse and rat(9-11). Gotoh has emphasized the importance of the stationary phase to avoid indiscriminate digestion of islets caused by prolonged agitation(19). Although islet yield is reportedly high, these studies did not compare yield in a strictly controlled fashion to the standard technique described by Lacy et al(2). Others have shown conflicting results with no improvement in islet yield in the rat(3); Van Suylichem et al mimicked the pattern of collagenase distribution after intraductal delivery using India Ink, and found permeation not only of the islet peri-insular space, but invasion of peripheral islet endocrine cells, of islet capillaries and of the islet core, irrespective of the route of administration(3).

In developing modifications to stationary digestion for optimal islet isolation in the rat, we identified several critical factors that improved consistency of high islet yield. If the pancreas was perfused with arterial blood at the time of ductal collagenase injection, a severe hemorrhagic pancreatitis developed, predominantly in the tail of the gland, preventing satisfactory islet liberation; the hemorrhagic pancreatitis presumably activated autolytic acinar enzymes leading to islet destruction. Unless the integrity of the pancreatic capsule was maintained during distention, islet yield deteriorated. We found that gentle pulsed ductal collagenase injection, coupled with splenic elevation, allowed maximal glandular distention, and mechanical disruption of the islet-acinar interface, with optimal islet liberation. Thus a more dilute collagenase solution could be used for digestion, reducing islet loss from over-digestion.

Storage of collagenase distended pancreata on ice for 30 minutes was found to have significant beneficial impact on islet recovery. Although difficult to understand why this step is effective, it may be that a period of cold storage in collagenase allows autolytic activation of endogenous pancreatic enzymes that might assist in the overall digestion process. Additionally there may be some residual slow activity of collagenase itself at +4∞C.

Careful standardization of the use of an electronic vortex for post-digestive glandular dissociation permitted further liberation of trapped islets and improved islet recovery.

Comparing the modified stationary digestion (*Stationary*) to standard chopped tissue digestion (*Chopped*) we have clearly shown that significant improvement in islet recovery is achieved. Islets isolated by *Stationary* demonstrated biphasic insulin release in vitro on dynamic perifusion, not significantly different from *Chopped* isolations. There was a trend towards improved phase II insulin release, but mild impairment in insulin 'switch-off' after hyperglycemic challenge. This most likely reflected a degree of recoverable beta cell membrane damage induced by a prolonged warm ischemic digestive phase (35 minutes) compared to *Chopped* (11 minutes). After isogeneic transplantation of *Stationary* islets, in vivo function was satisfactory, with successful reversal of the diabetic state in all recipients. Early graft function was modestly impaired in the *Stationary* group (3.3 vs 1.6 days to restore normoglycemia for *Chopped*), again reflecting reversible warm ischemic injury. At one month, function on IVGTT in all islet transplants was excellent, with identical function to that of normal non-diabetic controls. Graft-bearing nephrectomy led to immediate hyperglycemia in all animals, confirming the reliability of the streptozotocin model of diabetes.

Economic assessment proved that modified stationary digestion was cost effective, allowing substantial saving in the use of collagenase and donor rats.

We conclude that modified stationary digestion significantly improves islet recovery compared to standard chopped tissue digestion techniques. Excellent in vitro and in vivo graft function has been demonstrated, despite a prolonged warm digestion phase. Our modifications to stationary digestion have provided a simplified but reliable isolation technique which is cost effective.

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CHAPTER 6:

DEVELOPMENT AND RATIONALE FOR IMPLEMENTATION OF THE "EDMONTON PROTOCOL"

6.1 DEVELOPMENT OF THE "EDMONTON PROTOCOL" – EXTRAPOLATION FROM BASIC TO APPLIED CLINICAL SCIENCE

To move from the basic studies outlined to this point in the thesis to a new clinical trial (initiated in 1999) demanded implementation of several steps that were not clearly supported by all of the preceding pre-clinical data. These steps require clear justification, as outlined below.

A careful review of all clinical outcomes in islet transplantation defined in this thesis up to this point, indicated the following:

1. Cyclosporine and glucocorticoid-based immunosuppression was ineffective in controlling acute rejection in islet transplantation, with patients only rarely attaining insulin independence, and C-peptide secretion diminishing to zero over time in the majority of cases(*1, 2*).
2. Patients with end-stage renal failure may be a challenging group of patients to transplant with islets, because of pre-existing insulin resistance induced by the uremic state which only slowly reverts over time(*3*).
3. It was clear from the registry data that many patients were unable to reach or maintain insulin independence because: i) the islet infusion mass was sub-therapeutic, ii) a high proportion of the infused islet mass was failing to engraft, iii) islets were being damaged by direct local toxic effects from the immunosuppressants, or iv) ineffective immunosuppression failed to prevent acute or chronic rejection, or recurrence of autoimmune diabetes(*2*). Up to 50% of the infused islet mass may be destroyed through apoptotic and other non-immune inflammatory pathways, including immediate non-specific blood-mediated platelet binding and activation, leading to islet destruction(*4*).

4. A thorough review of state-of-the-art immunosuppression (chapter 2) indicated that promising new therapies were rapidly being introduced into the clinic – providing a unique opportunity to design more potent but less diabetogenic regimens specifically tailored to meet the needs of an islet graft. Of these therapies, the drug sirolimus appeared to be most promising.
5. Previous studies by Yakimets *et al* in experimental islet transplantation suggested that the combination of sirolimus and cyclosporine might be effective for more optimal control of rejection(*5*). The design of these preliminary studies involved complete immunosuppressive withdrawal after one month, limiting interpretation of longer-term potential efficacy.
6. Kneteman *et al* adapted the frequently sampled glucose tolerance test and Minimal Model kinetics for intrasplenic islet autografts, and clearly demonstrated that sirolimus was not diabetogenic – and in fact improved islet graft function in part through a 13% decrease in insulin clearance rates leading to prolongation in insulin half-life(*6*). The combination of cyclosporine and sirolimus was promoted for its reduced diabetogenic potential in this islet autograft model.
7. Experiments discussed in detail in Chapter 3 of this thesis clearly demonstrated that the combination of glucocorticoids with calcineurin inhibitors led to profound impairment of islet autograft function, and in this situation the tacrolimus + glucocorticoid combination was far more damaging to islet function than cyclosporine + glucocorticoid.
8. Experiments reported in Chapter 4 demonstrated the concept of the "portal immunosuppressive storm" – of potential concern to an islet graft recently embolized to an intrahepatic site. These experiments further suggested that

an intrasplenic islet autograft model might underestimate the degree of diabetogenicity since this graft site is spared from the "portal storm" effect after oral drug delivery.

As a result of the above discussion, it was felt appropriate to further explore the potential of sirolimus + cyclosporine in a series of canine islet allografts. These experiments were completed, and the results are illustrated in Figure 6.1 below:

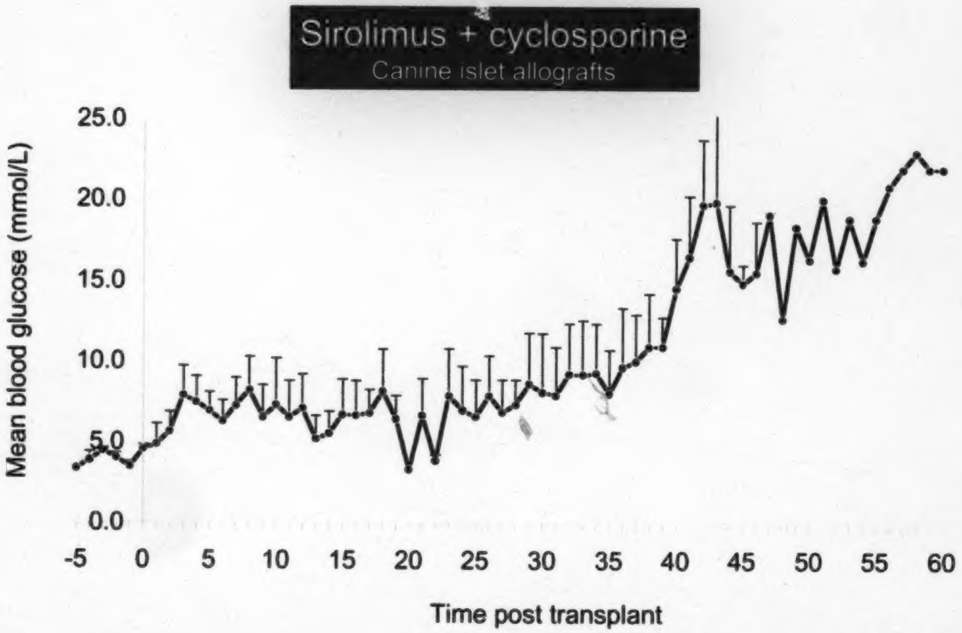


Figure 6.1: Mean blood glucose after intrasplenic canine islet allograft transplantation with sirolimus + cyclosporine immunosuppression. The combination of sirolimus with cyclosporine failed to prevent graft failure, and ALL canine islet allografts (n=8) failed within one month, despite level controlled therapeutic drug monitoring for cyclosporine (400-600µg/L) and sirolimus (20-25µg/L)

The outcome of these studies was disappointing, and contrasted with the previously encouraging results of Yakimets *et al* in a similar model(*5*), but where therapy was continued indefinitely unlike in the previous study where therapy was terminated at 30 days. In all cases (n=8) normoglycemia was attained immediately post transplant, confirming success of the islet isolation and engraftment procedure. However all grafts were rejected within 3-4 weeks, despite intensive therapeutic drug level monitoring for both cyclosporine and sirolimus. Indeed, levels were maintained at 400-600µg/L for cyclosporine (as in the previous study, targeting trough drug levels at the upper end of the clinical therapeutic range), and at 20-25µg/L for sirolimus (HPLC). Histological evaluation of the intrasplenic grafts failed to identify the presence of insulin positive cells once hyperglycemia had been present for more than seven days. The negative outcome of this study dissuaded further exploration of this combination in the clinical setting. Furthermore, while initial clinical studies of sirolimus + cyclosporine + glucocorticoids reported rejection rates of 7.5%(*7*), a larger controlled trial demonstrated rejection rates of 16-19%(*8, 9*). These rates would have likely translated to a 16-19% islet graft loss from rejection, and a higher primary failure rate due to the diabetogenic impact of cyclosporine and glucocorticoids in that regimen. Additional concerns related to a potential for enhanced nephrotoxicity, dyslipidemia and elevated blood pressure due to potential interactions between sirolimus and cyclosporine that may intensify the action of cyclosporine(*8, 9*).

It was clear at this point that sirolimus + cyclosporine + glucocorticoid therapy would not be an ideal combination for a new clinical islet transplant trial, although results may have improved upon the cyclosporine + glucocorticoid + azathioprine as used previously by others. It was also apparent from our preliminary experience with high dose sirolimus (level controlled at 20-25 µg/L) with short-term glucocorticoid therapy in clinical liver transplantation that rejection rates of more than 20% were not ideal for the islet transplant situation.

Consideration was therefore given to the use of sirolimus in combination with low dose tacrolimus to further enhance immunologic efficacy. Tacrolimus-based immunosuppression has proved to be consistently more effective in whole pancreas transplantation than cyclosporine in prophylaxis of rejection(*10*). Furthermore, the most successful reported outcomes in islet allograft transplantation were in cluster liver-islet cases immunosuppressed with tacrolimus (and without glucocorticoids in some cases) - although it was uncertain whether the early success reflected better immunosuppression or avoidance of the autoimmune environment(*11*).

Up to this point it was generally regarded that tacrolimus could not be given in combination with sirolimus because of competitive binding to the FKBP12 and FKBP25 binding proteins(*12*). However studies reported by Chen and Vu in rodent heart and small bowel allograft studies revealed that this interaction was not seen when sirolimus and tacrolimus were given at clinically relevant rather than pharmacologically saturating dose, encouraging us to explore this combination further(*13, 14*). Shortly thereafter, preliminary data from the Halifax group began to emerge, suggesting that sirolimus + low-dose tacrolimus + early glucocorticoid therapy could be used safely in patients(*15*). Furthermore the reported rejection rates were unprecedented in any previous clinical transplant trial, and have now remained so in longer follow up studies(*16, 17*).

The canine islet autograft studies (Chapter 3) strongly implied that a glucocorticoid-free immunosuppressant regimen would be an essential feature to promote success of a new clinical islet study, and that tacrolimus + glucocorticoid caused irreversible destruction in all canine islet autografts. This information precluded direct application of the protocol used by the Halifax group. It was not known at this juncture whether complete elimination of glucocorticoids from the sirolimus + low-dose tacrolimus regimen would result in adequate immunologic efficacy. Therefore this combination was evaluated in a further series of canine islet allografts, with the following results (Figure 6.2):

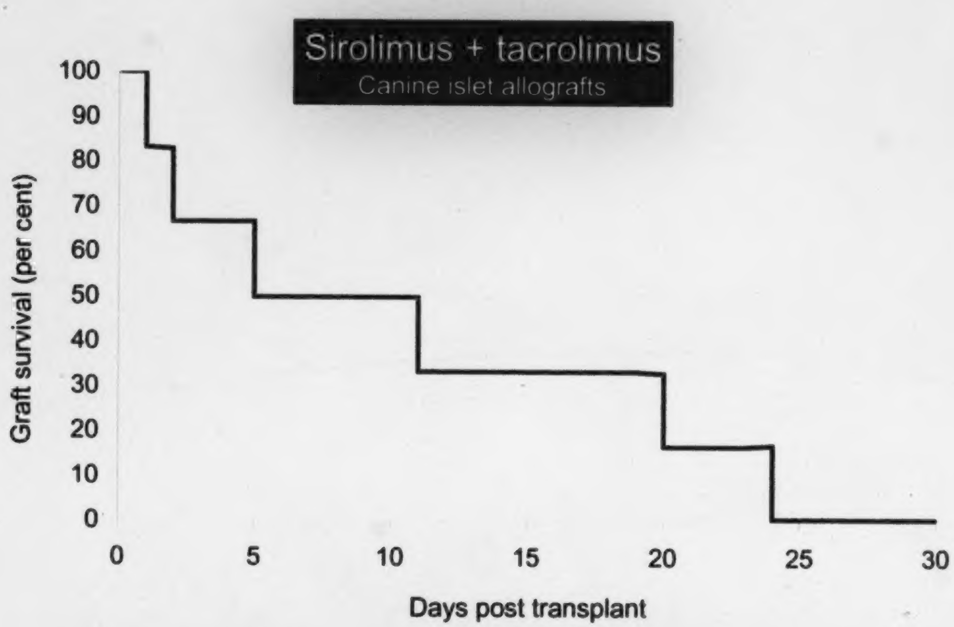


Figure 6.2: Kaplan-Meyer survival curve – canine intrasplenic islet allografts under the cover of combined sirolimus + low dose tacrolimus. All grafts failed (n=6), or animals succumbed from drug-related toxicity within 24 days (median survival 8.5 days)

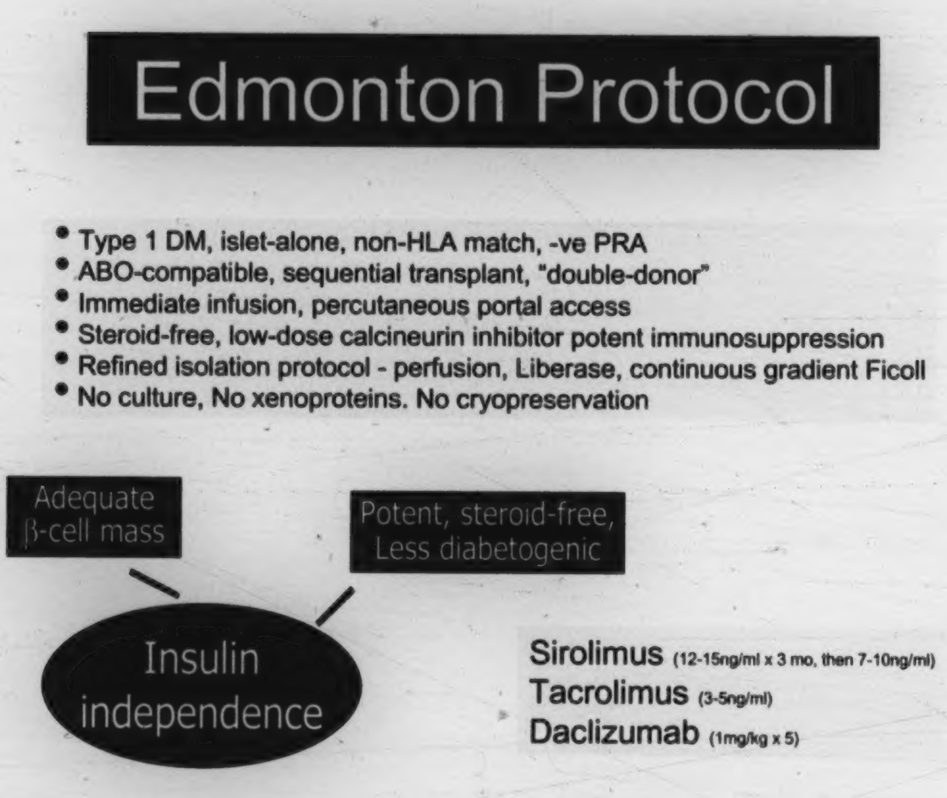
The combination of sirolimus with low-dose tacrolimus was extremely poorly tolerated in the dog model. Three animals succumbed within five days of transplantation from liver failure and disseminated intravascular coagulopathy. This may have been related to the use of unpurified islet preparations infused in retrograde fashion into the splenic vein in this group. Total vascular clamp occlusion of the main splenic pedicle was in effect during islet infusion, and was maintained for at least five minutes post infusion, as recommended previously(*18*). Delayed graft transmigration to the portal vein may have exacerbated this outcome. Three further grafts failed through presumed acute rejection occurring on day 11, 20 and 24 respectively. Target sirolimus levels of 20-25 µg/L and tacrolimus levels of 5-10 µg/L were used for this study.

Had the outcome message of this study been adhered to according to the usual scientific manner, there might never have been an "Edmonton Protocol." While the canine islet allograft study was ongoing, a 45-year-old teacher with longstanding Type 1 diabetes presented in severe recurrent coma from hypoglycemic. A high yield, blood group compatible islet preparation became available on 11th March 1999, and islet transplantation proceeded under what has now been termed the "Edmonton Protocol." This protocol has since proven to be highly effective in the clinical islet transplant setting (Chapter 7), providing an unprecedented high success rate in terms of insulin independence after islet alone transplantation in Type 1 diabetes. The changes made to the clinical protocols together with procedural changes for islet isolation methods applied in the "Edmonton Protocol" are outlined and justified below:

The major changes to the previous protocol involved:

1. Potent immunosuppression without glucocorticoid exposure, using daclizumab induction with maintenance sirolimus and low-dose tacrolimus.
2. Immediate transplantation of freshly isolated islets, without culture or use of cryopreserved tissue
3. Replacement of xenoproteins used previously during islet isolation (fetal calf serum) with more compatible human albumin solutions.
4. Focus on a new group of patients without renal failure, for islet-alone transplantation

5. Use of "double-donor" transplants to provide an adequate therapeutic islet mass to sustain independence from insulin.
6. Modifications made to the previous islet isolation techniques (controlled ductal perfusion, use only of low-endotoxin containing, purified collagenase blends (Liberase™), and top-loaded continuous-gradient commercial Ficoll solutions (Seromed™).
7. A multidisciplinary team-approach that facilitated rapid development and implementation of the new protocol.



Edmonton Protocol

- Type 1 DM, islet-alone, non-HLA match, -ve PRA
- ABO-compatible, sequential transplant, "double-donor"
- Immediate infusion, percutaneous portal access
- Steroid-free, low-dose calcineurin inhibitor potent immunosuppression
- Refined isolation protocol - perfusion, Liberase, continuous gradient Ficoll
- No culture, No xenoproteins. No cryopreservation

Adequate β-cell mass

Potent, steroid-free, Less diabetogenic

Insulin independence

Sirolimus (12-15ng/ml x 3 mo, then 7-10ng/ml)
Tacrolimus (3-5ng/ml)
Daclizumab (1mg/kg x 5)

Figure 6.3: Summary of key modifications defined as the "Edmonton Protocol."

6.2 IMMUNOSUPPRESSION

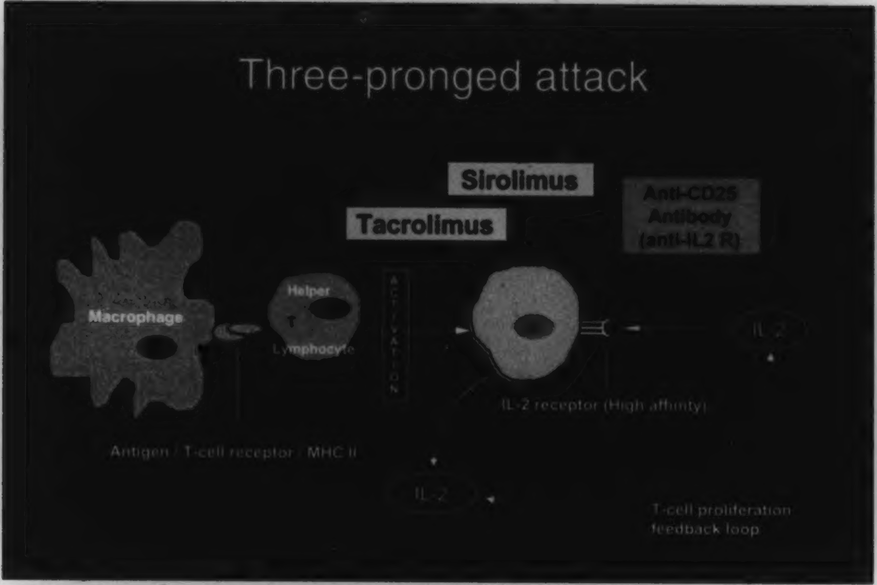
Regarding the transition to glucocorticoid-free immunosuppression, the ultimate decision to use the combination of sirolimus, low-dose tacrolimus and an anti-IL2R mAb was built on a synthesis of knowledge and experience with clinical use of each of these drugs in whole pancreas and liver transplantation, but clearly the underlying principles were driven by the over-riding lessons from the pre-clinical studies outlined in this thesis.

In the "Edmonton Protocol" glucocorticoids were eliminated because of risk of diabetogenicity defined above. Based on encouraging reports of the beneficial impact of anti-IL2R mAb therapy in renal transplantation(*19*, *20*), and lower rejection rates and preliminary data suggesting improved outcome in whole pancreas transplantation (Stephen T Bartlett, University of Maryland, personal communication), an empiric decision was made to replace glucocorticoids with an anti-IL2R mAb antibody. This innovative strategy was designed to provide potent synergistic immunosuppression in the absence of glucocorticoids, thereby avoiding diabetogenic impact on a limited beta-cell transplant reserve. Furthermore, it allowed other higher-risk induction therapies such as OKT3 to be eliminated from the regimen. Multivariate analyses from the Islet Transplant Registry had clearly revealed previously that use of OKT3 with its associated first-dose systemic cytokine release syndrome was detrimental to islet functional survival and outcome(*21*). These clinical observations were corroborated by previous experimental observations by Rabinovitch *et al* showing detrimental impact of local cytokines on islet survival(*22*).

Of the two available anti-IL2R mAb, daclizumab was felt to offer the following advantages over basiliximab for a clinical islet transplant trial: 1) The recommended therapeutic course involved 5 doses distributed across a 10 week period, thereby providing a longer window of 'induction' coverage for a subsequent transplant; 2) the daclizumab antibody is humanized and not chimeric, thereby reducing the risk of incompatibility, and 3) preliminary clinical reports suggested adequate safety and efficacy with extended dose regimens of daclizumab therapy in psoriasis patients; these studies have recently been published(*23, 24*).

The anti-IL2R was given pre-transplant at 1mg/kg pre-transplant, then repeated at two weekly intervals post-transplant for a total of 5 doses)(*25, 26*). In cases where a second islet graft was given beyond the 10-week induction window, the induction course of daclizumab was repeated. Sirolimus was given as a loading dose of 0.2mg/kg orally immediately pre-transplant, with maintenance initially at 0.1mg/kg/day adjusted to 24-hour target serum trough levels of 12-15ng/ml for three months, then reduced to 7-10 µg/L thereafter (measured by HPLC). Low-dose tacrolimus was begun at 2mg orally given twice daily, but adjusted to target 12-hour trough levels of 3-6 µg/L – representing between one quarter and one half of the usual standard dose for other transplants. The further aim was to maintain effective immunosuppression while minimizing the risk of tacrolimus-related islet injury(*27*).

Extremely low rejection rates with maximal control of autoimmunity remain essential prerequisites for islet cell transplantation in the absence of reliable markers of early rejection. The combined therapeutic strategy was developed in a concerted effort to prevent activation of the immune cascade by inhibiting: i) T-cell activation, ii) IL-2 and other recruitment cytokine production, iii) IL-2 receptor-ligand engagement, and iv) "Signal 3" proliferation with clonal expansion (Figure 6.4)(*8*).



Three-pronged attack

Sirolimus

Tacrolimus

Anti-CD25
Antibody
(anti-IL2 R)

Macrophage

Helper
T
Lymphocyte

ACTIVATION

IL-2

IL-2 receptor (High affinity)

Antigen / T-cell receptor / MHC II

IL-2

T-cell proliferation
feedback loop

Figure 6.4: Tri-site combination therapy to prevent rejection and control autoimmunity in the absence of glucocorticoids.

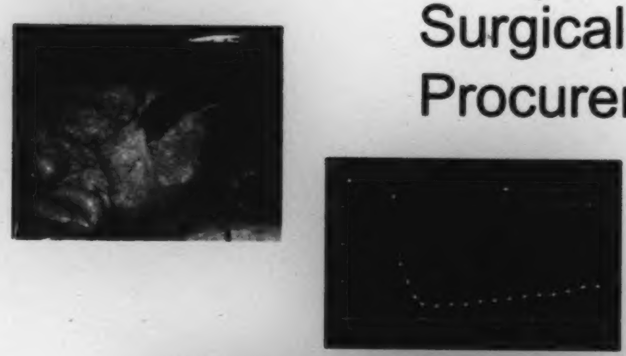
6.3 CHANGES IN ISLET ISOLATION PROCEDURES – EVOLUTION IN THE STATE OF THE ART

Recent progress has occurred in the science of islet isolation, based on evolution of an enzymatic pancreatic dissociation process that provides more consistent high yields of viable human islets for transplantation. The techniques used currently evolved in a strong international collaborative effort with a select number of islet isolation laboratories. The history and development of the current state of the art isolation methods have been reviewed in detail in Chapter 1. Recent methods have increased the efficiency of the process, and have had major impact in enhancing the consistency and quality of highly purified islet preparations for safe transplantation into patients.

6.3.1 PANCREAS PROCUREMENT

Human pancreases were recovered from brain dead cadaveric donors and stored in chilled University of Wisconsin (UW) solution after obtaining informed consent. Appropriate donor selection followed guidelines from a recent multivariate analysis of factors determining success of islet isolation(*28*). The following principles of pancreas procurement were adhered to wherever possible: 1) atraumatic pancreatic handling, 2) rapid *in situ* cooling after donor aortic cross-clamping by pancreatic mobilization and surrounding the pancreas with iced saline slush within the lesser sac to minimize warm ischemic insult, 3) minimal cold ischemic injury by rapid transfer of the excised pancreas to the islet isolation laboratory. Studies completed collaboratively with Dr Jonathan Lakey revealed that rapid mobilization of the spleen to midline at the time of aortic cross-clamp, and embedding of the entire pancreas in iced slush-saline led to a doubling in islet yield with associated significant improvement in islet viability (Figure 6.5)

Surgical Procurement



	Yield	Function (SI)
Ice:	223 ± 35 IE x10^3	4.4 ± 0.3
No ice:	103 ± 26 IE x10^3	3.0 ± 0.4
	$p<0.01$	*$p<0.05$*

Figure 6.5: Impact of surgical procurement technique on islet yield and functional viability.

(Red line – core pancreatic temperature where no ice was placed in lesser sac vs yellow line – pancreas embedded in iced saline during multivisceral retrieval. Study conducted in collaboration with Dr Jonathan Lakey)

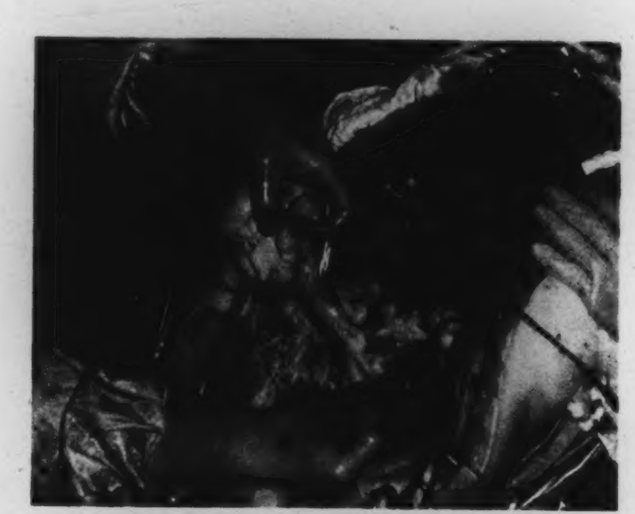


Figure 6.6: Pancreas is removed *en bloc* with stapled duodenum and spleen, ensuring that pancreatic capsule is maintained intact.

More rigorous selection criteria of suitable pancreata for processing and potentially individualized isolation protocols based on the several donor variables identified may improve consistency in human islet isolation and thereby decrease costs(*28*).

6.3.2 ISLET ISOLATION AND PURIFICATION

Islets were isolated using previously described techniques of controlled enzymatic ductal perfusion, digestion and Ficoll purification(*29, 30*). A highly purified collagenase enzyme blend (Liberase™ HI, Boehringer Mannheim, Indianapolis, IN) was used for pancreatic dissociation, to minimize endotoxin exposure and to maximize islet yields(*31-34*).

6.3.3 PANCREATIC DUCTAL PERFUSION

One vial of Liberase™-HI enzyme was re-suspended in 30 ml of cold Hank's balanced salt solution, HBSS (Gibco, Grand Island, New York), allowed to rehydrate for 30 min before being brought up to a volume of 350 ml in HBSS containing 25 mmol HEPES, and sterile filtered using a 0.22 µm cellulose acetate filter (Corning) (final Liberase™ concentration 1.43mg/ml). The pancreas was then cleaned of covering fat, and the attached duodenal loop was dissected free. Surface antibiotic and antimycotic decontamination was then carried out by a five minute exposure to a solution of 30mls of Betadine 80 mg gentamicin, 1g Cefazolin and 100 mg amphotericin-B in a volume of 150 ml of cold HBSS, followed by rinsing in cold HBSS. After 5-minute incubation, the pancreas will be serially rinsed in two beakers containing 500mls of HBSS. Two cannulae (Medicut 14-18 gauge catheter) were then inserted into the main pancreatic duct and directed towards the head and tail of the pancreas and secured in place(*35*).

Diluted Liberase™ enzyme solution was then perfused through the main pancreatic ducts, while maintaining constant pressure of 60-80 mmHg for the first 5 min and 160-180 mmHg for the next 5 min(*35*). After 10 min of cold perfusion, the distended pancreas was then cut into segments and transferred to a Ricordi-type dissociation chamber(*30*).

6.3.4 PANCREATIC DISSOCIATION

After transfer to the dissociation chamber, the recirculating collagenase solution was warmed slowly to 37°C and held at 37±0.5°C throughout. The chamber was shaken gently while the tissue dissociated, and aliquots of pancreatic tissue were evaluated during the digestion phase by staining with Dithizone™. Once the majority of islets were free from exocrine tissue the digestion phase was stopped, and warm (22-37°C) MEM solution was flushed through the chamber with the effluent collected in 250 ml Corning tubes containing 15 ml of 25% human albumin. All tissue was then recombined and a pre-purification sample removed, stained with Dithizone™ and assessed as described previously(*36*). The tissue digest was incubated in a volume of 100 ml of cold UW solution for a period of 30-45 minutes prior to purification.

6.3.5 PURIFICATION AND ISLET QUANTIFICATION

Pancreatic digest was purified using continuous gradients of Seromed™ Ficoll, as originally described by the Giessen Group, using the Cobe 2991 (COBE BCT, Inc., Lakewood, CO)(*37*). Samples of several layers were removed, stained with Dithizone™ and assessed for islet purity. Islet enriched layers were selected and collected. Islet recovery following purification was assessed in duplicate by counts of Dithizone™-stained aliquots of the final tissue suspension.

The islet mass was quantified and the purity of the preparations assessed by two independent investigators in accordance to the criteria established at the 1989 International Workshop on Islet Assessment(*36*). The number of islets in each diameter class was determined using an optical graticule. The number of islets within each size class was then converted to the standard number of IE that represents the number of islets of 150 µm diameter equal in volume to the sample. Purity of the preparations was assessed by comparing the relative quantity of Dithizone™-stained endocrine tissue with unstained exocrine tissue. Immediately prior to transplantation, islet sample aliquots were collected for retrospective microbiological culture, and for *post hoc* determination of islet graft characterization. Islet preparations were then re-suspended in a solution of 20% human albumin and MEM media without phenol red, in preparation for transplantation.

Xenoprotein products (fetal calf serum, etc) were not used during any stage of the islet isolation or purification procedure. In order to minimize islet injury from cold ischemia, immediate transplantation of freshly prepared islets was carried out, thus completely eliminating the islet culture stage that was previously standard in most islet transplant centers. By obviating a need for tissue culture, there was no need to supplement the media with xenoprotein tissue culture growth factors. Non-specific islet protein coating could theoretically be one target for immediate islet destruction after human transplantation(*4*). All xenoprotein products used during dilution and wash steps of islet processing were therefore substituted for human albumin solution.

The ability to consistently isolate a higher quality and quantity of islets from human pancreata has been a key to the development and continuation of experimental and clinical trials in islet transplantation as a realistic treatment option for patients with type 1 diabetes.

6.4 BALANCING THE RISK-BENEFIT RATIO FOR ISLET-ALONE TRANSPLANTS

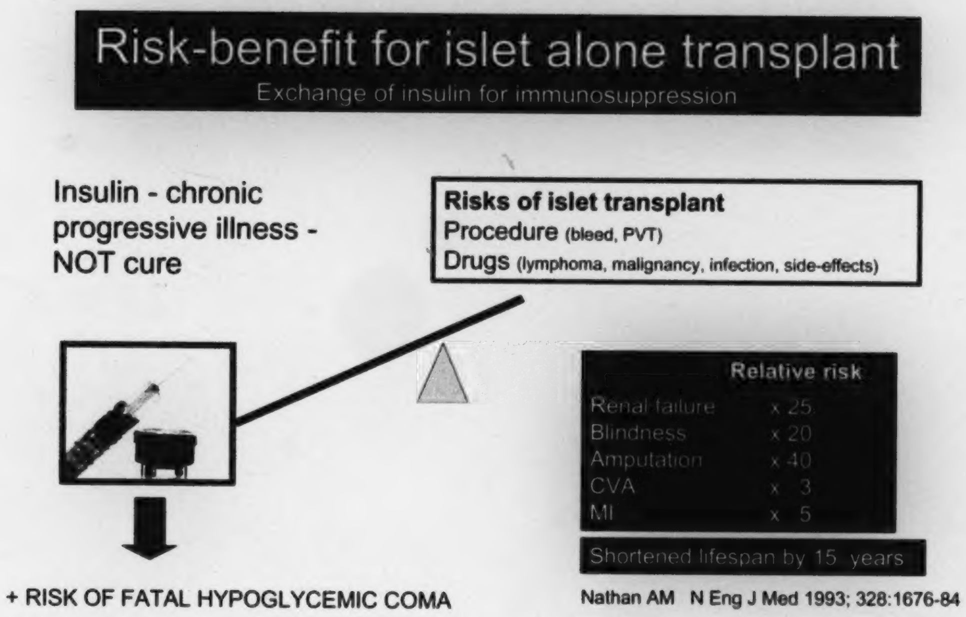
While insulin injection therapy is life sustaining for patients with Type 1 diabetes, the condition remains a chronic illness, with most patients developing one or more end-stage complications over time. Compared with unaffected individuals, patients with diabetes have a 25 times increased risk of renal failure, 20 times the risk of blindness, 40 times the risk of amputations, 3 times the risk of stroke, and 5 times the risk of myocardial infarction(*38*). This results in an expected lifespan that is shortened by an average of 15 years compared with the general population(*38*). Thus diabetes is not cured by insulin, and is ultimately dangerous to most sufferers (Figure 6.7)

In highly selected patients with severe hypoglycemic coma, unawareness or marked metabolic lability, the long-term risks of diabetes are further compounded by shorter-term risks of fatal events, including the "dead-in-bed" syndrome(*39*). In these individuals it is not hard to justify the exchange of insulin for immunosuppression following islet transplantation. Potential risks of islet transplantation are outlined below:

6.5 THE PROCEDURE

6.5.1 BLEEDING

Bleeding from the liver puncture site may occasionally require blood transfusion after percutaneous transhepatic access to the portal vein(*40*). This risk may be reduced by using lower amounts of systemic anticoagulation at the time of transplantation, by use of thrombostatic agents delivered down the catheter tract on completion of the infusion, or by using a smaller catheter caliber (e.g. O'Kelly 4Fr islet transplant catheter, Cook Inc., with an inner lumen diameter of 900µm). Alternative routes of portal access including trans-jugular intrahepatic techniques (adapted from the TIPPS procedure), may diminish the risk of bleeding, but may be more challenging and will prolong the duration of the procedure.



Risk-benefit for islet alone transplant

Exchange of insulin for immunosuppression

Insulin - chronic progressive illness - NOT cure

Risks of islet transplant
Procedure (bleed, PVT)
Drugs (lymphoma, malignancy, infection, side-effects)

	Relative risk
Renal failure	x 25
Blindness	x 20
Amputation	x 40
CVA	x 3
MI	x 5

Shortened lifespan by 15 years

+ RISK OF FATAL HYPOGLYCEMIC COMA

Nathan AM N Eng J Med 1993; 328:1676-84

Figure 6.7: Balancing the risk-benefit ratio in favor of islet transplantation in highly selected individuals with failure of intensive insulin – justifying the exchange of insulin for immunosuppression.

6.5.2 PORTAL VEIN THROMBOSIS

Portal vein thrombosis has been described rarely in the past, and was often associated with transplantation of impure or partially purified islets(*41*). Before the advent of low-endotoxin containing collagenase enzyme preparations and the availability of Ficoll-based islet purification gradients, the risk of portal thrombosis leading to diffuse intravascular coagulation, portal thrombosis, liver failure or even death have been described(*42*). These risks are perceived to be very low with highly purified islet preparations of packed cell volume less than 10cc's, and may be further minimized by careful monitoring or portal pressure during islet infusion.

6.5.3 TRANSMISSION OF INFECTION

Transmission of infection from the donor always remains a possibility, but thorough donor screening for hepatitis B and C, and for HIV viruses lowers the risks considerably. Cytomegaloviral (CMV) transmission has been a challenging problem in solid organ transplantation previously, and therefore intensive prophylaxis with oral ganciclovir was given to all recipients irrespective of mismatch between donor and recipient for CMV serology.

6.5.4 DRUG TOXICITIES

The risk of renal dysfunction due to calcineurin inhibition has been diminished recently with the evolution of low-dose tacrolimus based regimens. Gastrointestinal side effects of tacrolimus are not infrequent, and may lead to episodic diarrhea. Neutropenia from sirolimus, and the risk of mouth ulceration may be reduced by using lower target trough levels, and by using the tablet rather than a liquid formulation of sirolimus respectively. Neurotoxicity may be seen with tacrolimus, but is less frequent with low-dose regimens. Demyelination syndromes including central pontine myelinolysis are one of the most feared complications of tacrolimus, but fortunately is exceedingly rare with the highest risk seen in intensive care bound patients with severe hyponatremia undergoing liver transplantation.

6.5.5 LONG TERM RISKS OF IMMUNOSUPPRESSION

The risk of all types of malignancies are increased in chronically immunosuppressed individuals, but squamous epithelial cancers are the most common and most readily treatable. The lifetime risk of lymphoma is estimated to occur in 1-2% of transplant recipients. This risk may be an overestimate for islet recipients, where glucocorticoids are and exposure to OKT3 are avoided. However the long-term risks of sirolimus/low-dose tacrolimus based regimes are imprecisely known currently.

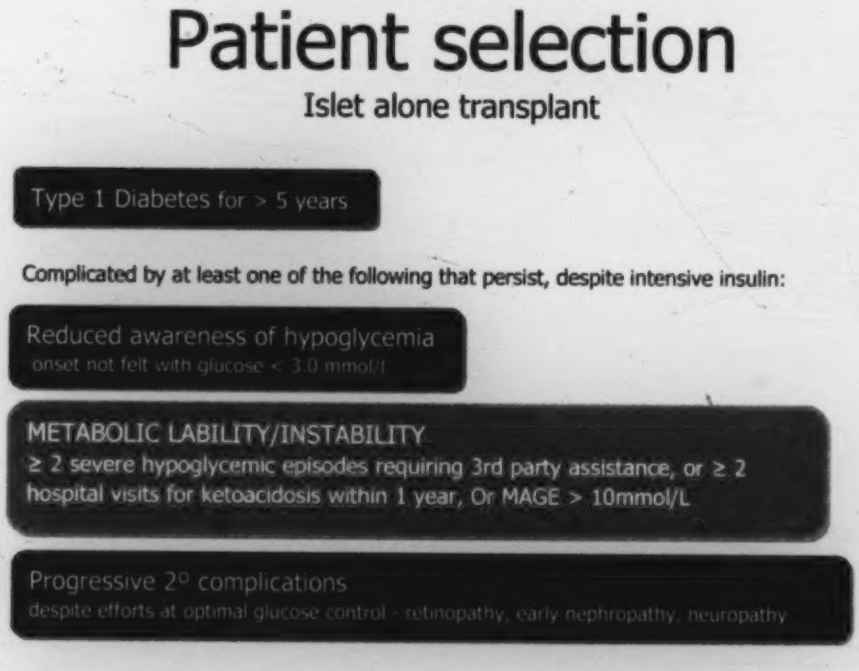
With the cumulative risk of the above procedural or immunosuppression-related complications, most patients with type-1 diabetes will be more safely managed with chronic insulin rather than immunosuppression at the present time. However in highly selected patients with type 1 diabetes compounded by frequent comas, brittle control or advancing complications, the risk-benefit ratio appears to fall strongly in favor of islet transplantation.

6.6 PATIENT SELECTION FOR ISLET-ALONE TRANSPLANTATION

Most islet transplants carried out previously were in combination with a kidney transplant in patients with end-stage diabetic renal failure(*43*). These individuals often have peripheral insulin resistance, which may slowly reverse after successful transplantation(*3, 44*). In the Edmonton trial, this problem was avoided by selecting C-peptide negative type-1 diabetic patients for islet-alone transplantation in the absence of renal failure(*40*). Patients were selected for islet-alone transplantation based on complications of hypoglycemic unawareness, metabolic instability or progression of secondary diabetic complications that persisted despite optimization of the insulin regimen, using similar criteria applied previously in solitary pancreas transplantation(*10*).

Three main groups of patients have been selected for islet-alone transplantation:

1. Patients with reduced hypoglycemic awareness,
2. Patients with brittle diabetes or labile diabetes, and
3. Patients with progressive complications



Patient selection

Islet alone transplant

Type 1 Diabetes for > 5 years

Complicated by at least one of the following that persist, despite intensive insulin:

Reduced awareness of hypoglycemia
onset not felt with glucose < 3.0 mmol/l

METABOLIC LABILITY/INSTABILITY
≥ 2 severe hypoglycemic episodes requiring 3rd party assistance, or ≥ 2 hospital visits for ketoacidosis within 1 year, Or MAGE > 10mmol/L

Progressive 2° complications
despite efforts at optimal glucose control - retinopathy, early nephropathy, neuropathy

Figure 6.8: Patient selection for islet alone transplantation

Clinical outcomes in the first seven patients are detailed in Chapter 7, and following this, a recent update in 18 consecutive patients is outlined in Chapter 8.

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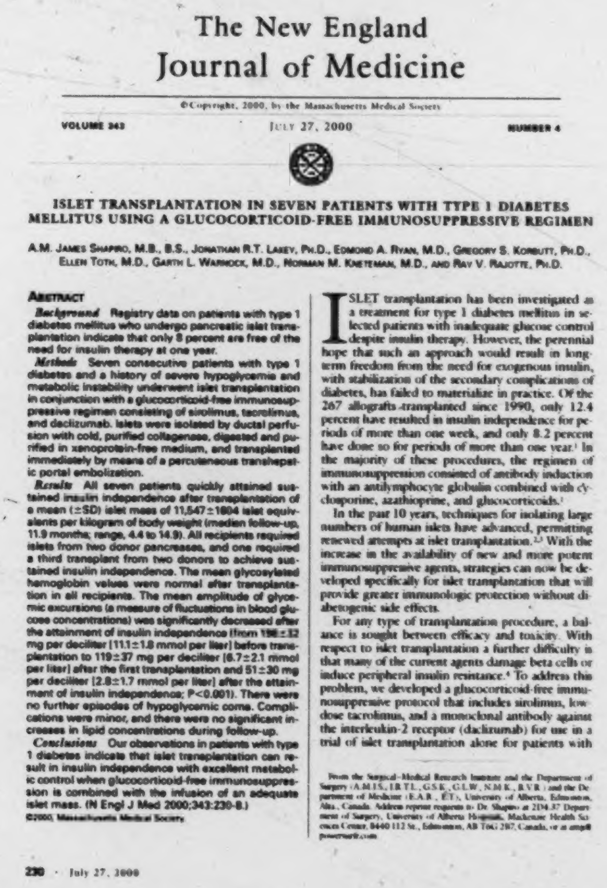
CHAPTER 7

ISLET-ALONE TRANSPLANTATION IN PATIENTS WITH TYPE 1 DIABETES MELLITUS USING A GLUCOCORTICOID-FREE IMMUNOSUPPRESSIVE REGIMEN



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ISLET TRANSPLANTATION IN SEVEN PATIENTS WITH TYPE 1 DIABETES MELLITUS USING A GLUCOCORTICOID-FREE IMMUNOSUPPRESSIVE REGIMEN

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ABSTRACT

Background Registry data on patients with type 1 diabetes mellitus who undergo pancreatic islet transplantation indicate that only 8 percent are free of the need for insulin therapy at one year.

Methods Seven consecutive patients with type 1 diabetes and a history of severe hypoglycemia and metabolic instability underwent islet transplantation in conjunction with a glucocorticoid-free immunosuppressive regimen consisting of sirolimus, tacrolimus, and daclizumab. Islets were isolated by ductal perfusion with cold, purified collagenase, digested and purified in xenoprotein-free medium, and transplanted immediately by means of a percutaneous transhepatic portal embolization.

Results All seven patients quickly attained sustained insulin independence after transplantation of a mean (±SD) islet mass of 11,547±1604 islet equivalents per kilogram of body weight (median follow-up, 11.9 months; range, 4.4 to 14.9). All recipients required islets from two donor pancreases, and one required a third transplant from two donors to achieve sustained insulin independence. The mean glycosylated hemoglobin values were normal after transplantation in all recipients. The mean amplitude of glycemic excursions (a measure of fluctuations in blood glucose concentrations) was significantly decreased after the attainment of insulin independence (from 198±32 mg per deciliter [11.1±1.8 mmol per liter] before transplantation to 119±37 mg per deciliter [6.7±2.1 mmol per liter] after the first transplantation and 51±30 mg per deciliter [2.8±1.7 mmol per liter] after the attainment of insulin independence; $P<0.001$). There were no further episodes of hypoglycemic coma. Complications were minor, and there were no significant increases in lipid concentrations during follow-up.

Conclusions Our observations in patients with type 1 diabetes indicate that islet transplantation can result in insulin independence with excellent metabolic control when glucocorticoid-free immunosuppression is combined with the infusion of an adequate islet mass. (N Engl J Med 2000;343:230-8.)

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ISLET transplantation has been investigated as a treatment for type 1 diabetes mellitus in selected patients with inadequate glucose control despite insulin therapy. However, the perennial hope that such an approach would result in long-term freedom from the need for exogenous insulin, with stabilization of the secondary complications of diabetes, has failed to materialize in practice. Of the 267 allografts transplanted since 1990, only 12.4 percent have resulted in insulin independence for periods of more than one week, and only 8.2 percent have done so for periods of more than one year.[1] In the majority of these procedures, the regimen of immunosuppression consisted of antibody induction with an antilymphocyte globulin combined with cyclosporine, azathioprine, and glucocorticoids.[1]

In the past 10 years, techniques for isolating large numbers of human islets have advanced, permitting renewed attempts at islet transplantation.[2,3] With the increase in the availability of new and more potent immunosuppressive agents, strategies can now be developed specifically for islet transplantation that will provide greater immunologic protection without diabetogenic side effects.

For any type of transplantation procedure, a balance is sought between efficacy and toxicity. With respect to islet transplantation a further difficulty is that many of the current agents damage beta cells or induce peripheral insulin resistance.[4] To address this problem, we developed a glucocorticoid-free immunosuppressive protocol that includes sirolimus, low-dose tacrolimus, and a monoclonal antibody against the interleukin-2 receptor (daclizumab) for use in a trial of islet transplantation alone for patients with

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Note: The original version of this article was first released by the New England Journal of Medicine on June 6th 2000, six weeks ahead of schedule, with the following note: "Because of its potential therapeutic implications, this article is being released before its publication date, in accordance with the Journal's policy (Angell M and Kassirer JP. The Ingelfinger Rule revisited. N Engl J Med 1991;325:1371-2)."The final version of the report was published on July 27th 2000. Chapter 8 also provides an update on 15 consecutive patients transplanted to date.

ABSTRACT

Background. Registry data on patients with type 1 diabetes mellitus who undergo pancreatic islet transplantation indicate that only 8 percent are free of the need for insulin therapy at one year.

Methods. Seven consecutive patients with type 1 diabetes and a history of severe hypoglycemia and metabolic instability underwent islet transplantation in conjunction with a glucocorticoid-free immunosuppressive regimen consisting of sirolimus, tacrolimus, and daclizumab. Islets were isolated by ductal perfusion with cold, purified collagenase, digested and purified in xenoprotein-free medium, and transplanted immediately by means of a percutaneous transhepatic portal embolization.

Results. All seven patients quickly attained sustained insulin independence after transplantation of a mean (±SD) islet mass of 11,547 ±1604 islet equivalents per kilogram of body weight (median follow-up, 11.9 months; range, 4.4 to 14.9). All recipients required islets from two donor pancreases, and one required a third transplant from two donors to achieve sustained insulin independence. The mean glycosylated hemoglobin values were normal after transplantation in all recipients. The mean amplitude of glycemic excursions (a measure of fluctuations in blood glucose concentrations) was significantly decreased after the attainment of insulin independence (from 198 ±32 mg per deciliter [11.1 ±1.8 mmol per liter] before transplantation to 119 ±37 mg per deciliter [6.7 ±2.1 mmol per liter] after the first transplantation and 51 ±30 mg per deciliter [2.8 ±1.7 mmol per liter] after the attainment of insulin independence; $P<0.001$). There were no further episodes of hypoglycemic coma. Complications were minor, and there were no significant increases in lipid concentrations during follow-up.

Conclusions. Our observations in patients with type 1 diabetes indicate that islet transplantation can result in insulin independence with excellent metabolic control when

glucocorticoid-free immunosuppression is combined with the infusion of an adequate islet mass.

7.1 INTRODUCTION:

Islet transplantation has been investigated as a treatment for type 1 diabetes mellitus in selected patients with inadequate glucose control despite insulin therapy. However, the perennial hope that such an approach would result in long-term freedom from the need for exogenous insulin, with stabilization of the secondary complications of diabetes, has failed to materialize in practice. Of the 267 allografts transplanted since 1990, only 12.4 percent have resulted in insulin independence for periods of more than one week, and only 8.2 percent have done so for periods of more than one year(*1*). In the majority of these procedures, the regimen of immunosuppression consisted of antibody induction with an antilymphocyte globulin combined with cyclosporine, azathioprine, and glucocorticoids(*1*).

In the past 10 years, techniques for isolating large numbers of human islets have advanced, permitting renewed attempts at islet transplantation(*2, 3*). With the increase in the availability of new and more potent immunosuppressive agents, strategies can now be developed specifically for islet transplantation that will provide greater immunologic protection without diabetogenic side effects. For any type of transplantation procedure, a balance is sought between efficacy and toxicity. With respect to islet transplantation a further difficulty is that many of the current agents damage beta cells or induce peripheral insulin resistance(*4*). To address this problem, we developed a glucocorticoid-free immunosuppressive protocol that includes sirolimus, low-dose tacrolimus, and a monoclonal antibody against the interleukin-2 receptor (daclizumab) for use in a trial of islet transplantation alone for patients with brittle type 1 diabetes. Most previous islet transplantations have been performed in combination with kidney transplantation in patients with end-stage diabetic nephropathy(*1*). We limited our procedure to islet transplantation alone and in doing so selected patients who had severe hypoglycemia (defined as multiple hypoglycemic episodes) or uncontrolled diabetes despite compliance with an insulin regimen.

7.2 METHODS

7.3.1 PATIENTS

Patients who were considered to have had type 1 diabetes for more than five years on the basis of a stimulated serum C-peptide concentration of less than 0.48 ng per milliliter (0.16 nmol per liter) were eligible to undergo islet transplantation if their serum glucose concentrations remained uncontrolled despite exogenous insulin therapy. Patients also had to have recurrent severe hypoglycemia with coma or metabolic instability to such an extent that the global risk of transplantation and immunosuppression was judged to be less than the risk of continued uncontrolled diabetes. All protocols were approved by the health research ethics board of the University of Alberta, and each patient gave written informed consent.

7.3.2 GLUCOCORTICOID-FREE IMMUNOSUPPRESSION

Immunosuppression was initiated immediately before transplantation. Sirolimus (Rapamune, Wyeth-Ayerst Canada) was given orally at a loading dose of 0.2 mg per kilogram of bodyweight, followed by a dose of 0.1 mg per kilogram per day, with monitoring of drug levels to maintain them in the range of 12 to15 ng per milliliter for the first three months and in the range of 7 to 10 ng per milliliter thereafter. Low-dose tacrolimus (Prograf, Fujisawa Canada) was given orally at an initial dose of 1 mg twice daily, and the dose was subsequently adjusted to maintain a trough concentration at 12 hours of 3 to 6 ng per milliliter (IMX enzyme immunoassay, Abbott). Daclizumab

(Zenapax, Roche Canada) was given intravenously at a dose of 1 mg per kilogram every 14 days for a total of five doses. If the second transplantation procedure occurred more than 10 weeks after the first, the course of daclizumab was repeated. No glucocorticoids were given at any time during the trial.

7.3.3 CONDITIONING REGIMEN AND POST-TRANSPLANTATION THERAPY

As soon as there were sufficient numbers of islets for transplantation, the patient was given intravenous antibiotics prophylactically (500 mg of vancomycin and 500 mg of imipenem), and oral supplementation with vitamin E (800 IU per day), vitaminB6 (100 mg per day), and vitamin A (25,000 IU per day) was initiated(*5*). Inhaled pentamidine (300 mg once a month) was given after transplantation to prevent infection with Pneumocystis carinii, and oral ganciclovir (1 g three times per day) was given for 14 weeks after transplantation irrespective of the patient's cytomegalovirus status to reduce the risk of graft loss(*6, 7*) and to protect against lymphoproliferative disorder(*8*).

7.3.4 ISLET PREPARATION

Pancreases were removed from brain-dead donors and stored in chilled University of Wisconsin solution after informed consent had been obtained from the donors' relatives. Donors were selected according to the results of a multivariate analysis of factors that influence the success of islet isolation(*9*). To isolate the islets, the ducts were perfused in a controlled fashion with a cold enzyme (Liberase human islet, Roche). The islets were then separated by gentle mechanical dissociation and purified with the use of continuous gradients of Ficoll-diatrizoic acid (Seromed-Biochrom) in an apheresis system (model 2991, Cobe Laboratories)(*2, 3, 10-13*). The use of xenoprotein products (such as fetal-calf serum) was avoided during islet isolation and purification, and 25 percent human albumin was used instead. To minimize the risk of islet injury as a result of cold ischemia, we transplanted freshly prepared islets immediately after harvesting them, thus eliminating the need for islet culture. Samples were collected in duplicate for the quantification of the islets, expressed in terms of islet equivalents, the standard unit for reporting variations in the volume of islets, with the use of a standard islet diameter of 150 μm(*14*). Islet grafts were characterized with respect to cell composition, total cellular insulin, DNA, and the extent of insulin secretion in vitro during a glucose challenge(*15*). In brief, the islets were incubated for24 hours at 37°C in CMRL 1066 medium with 10 percent fetal-calf serum and 25 mmol HEPES buffer. A known number of duplicate aliquots of islets were incubated in a low concentration of glucose (50 mg per deciliter [2.8 mmol per liter]) and a high concentration of glucose (360 mg per deciliter [20 mmol per liter]) for two hours, and the amount of insulin generated in response to the high-glucose challenge was divided by the amount generated by the low-glucose challenge to yield the mean insulin-release stimulation index.

7.3.5 ISLET TRANSPLANTATION

Islet preparations that had more than 4000 islet equivalents per kilogram of the recipient's body weight in a packed-tissue volume of less than 10 ml were judged safe for transplantation(*16*). Each islet preparation from a donor was matched to the recipient's blood type and cross-matched for lymphocytotoxic antibodies, but no attempt at HLA matching was made. Patients were sedated, and a percutaneous transhepatic approach was used to gain access to the portal vein under fluoroscopic guidance. Once access was confirmed, we used the Seldinger technique to place a 5-French Kumpe catheter within the main portal vein. Portal venous pressure was measured at base line and after islet infusion. The final islet preparation was suspended in 120 ml of medium 199 that contained 500 U of heparin and 20 percent human albumin and was infused over a period of five minutes. In all but the first 2 of the 15 procedures, on completion of the islet infusion, as the catheter was partially removed, gelatin-sponge (Gelfoam) particles were embolized into the peripheral catheter tract in the liver. Doppler ultrasonography of the portal vein and liver-function tests were performed within 24 hours after transplantation.

7.3.6 ASSESSMENT OF GLYCEMIC CONTROL AFTER TRANSPLANTATION

Insulin therapy was discontinued after each transplantation and was not resumed unless serum glucose concentrations rose above 200mg per deciliter (11.1 mmol per liter), in which case another transplantation was performed. Serum glucose concentrations were monitored by memory capillary glucose meters, and the resulting data were analyzed by computer (with Medisense and Precision Link software). To determine the extent of fluctuations in glucose concentrations in each patient, we measured the mean amplitude of glycemic excursions, which was calculated as the mean of the differences in the major fluctuations in high and low glucose values during two 24-hour periods(*17*); a minimum of seven measurements of capillary glucose were obtained (before a meal, two hours after a meal, at bedtime, and at 3 a.m.). The patients also underwent oral glucose-tolerance testing and mixed-meal testing. The homeostatic model assessment was used to calculate insulin sensitivity(*18*). We also measured glycosylated hemoglobin and serum C-peptide, creatinine, and lipid concentrations.

7.3.7 STATISTICAL ANALYSIS

Results are expressed as means ±SD or, in the case of nonparametric variables, as medians and ranges. Analysis of variance was conducted with use of the Sigmastat program.

7.4 RESULTS

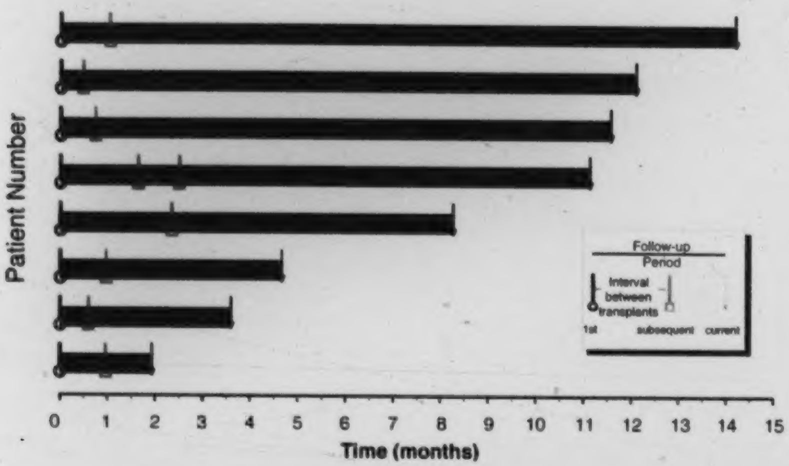

Figure 7.1: Length of follow-up after the initial transplant and the time at which subsequent transplantations were performed.

7.4.1 CHARACTERISTICS OF THE PATIENTS

Seven consecutive patients (median age, 44 years; range, 29 to 54) who had had type 1 diabetes mellitus for a median of 35 years (range, 18 to 50) underwent islet transplantation between March 11, 1999, and January 23, 2000. As of June 2000, the median duration of follow-up was 11.9 months (range, 4.4 to 14.9). In all seven patients, exogenous insulin therapy quickly became unnecessary once sufficient numbers of islets were transplanted. At the time of the most recent follow-up, all patients remained free of the need for exogenous insulin. The patient who received the smallest number of islets (Patient 1) has briefly required 4 to 10 U of insulin per day on four occasions during times of stress from intercurrent illness. One patient required a total of 7 U of insulin on a single occasion during a two-day illness. There have been no episodes of acute cellular rejection, as determined by measurements of glycemic control, serum insulin, and C-peptide. None of the patients have died. Six of the seven required a second islet infusion from a second donor pancreas a median of 29 days (range, 14 to 70) after the first procedure (Figure 7.1) to become insulin independent. One patient, the most obese (weight, 93 kg), required a third infusion to achieve insulin independence. The third infusion combined islets from two donors because of mechanical failure in one of the purification runs. All patients had had repeated episodes of severe hypoglycemia before transplantation but have had no further episodes since transplantation. This change has dramatically improved their quality of life. The mean (±SD) total number of islets required to induce insulin independence was 11,547 ±1604 islet equivalents per kilogram of the recipient's body weight, with a mean total beta-cell mass per transplant of 132 ±67 x106 (Table 7.1below).

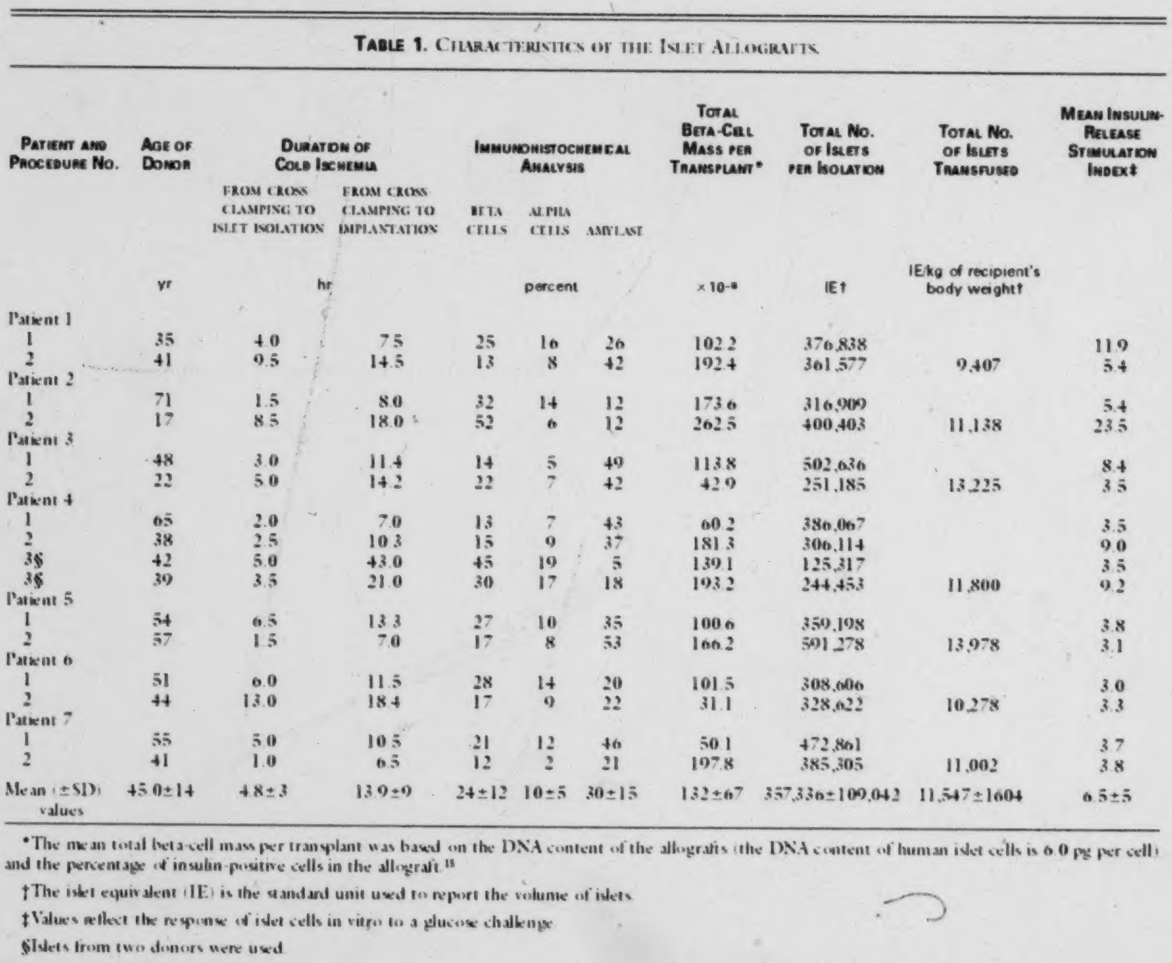

TABLE 1. CHARACTERISTICS OF THE ISLET ALLOGRAFTS.

PATIENT AND PROCEDURE NO.	AGE OF DONOR	DURATION OF COLD ISCHEMIA		IMMUNOHISTOCHEMICAL ANALYSIS			TOTAL BETA-CELL MASS PER TRANSPLANT*	TOTAL NO. OF ISLETS PER ISOLATION	TOTAL NO. OF ISLETS TRANSFUSED	MEAN INSULIN-RELEASE STIMULATION INDEX‡
		FROM CROSS CLAMPING TO ISLET ISOLATION	FROM CROSS CLAMPING TO IMPLANTATION	BETA CELLS	ALPHA CELLS	AMYLASE				
	yr	hr		percent			$\times 10^{-6}$	IE†	IE/kg of recipient's body weight†	
Patient 1										
1	35	4.0	7.5	25	16	26	102.2	376,838		11.9
2	41	9.5	14.5	13	8	42	192.4	361,577	9,407	5.4
Patient 2										
1	71	1.5	8.0	32	14	12	173.6	316,909		5.4
2	17	8.5	18.0	52	6	12	262.5	400,403	11,138	23.5
Patient 3										
1	48	3.0	11.4	14	5	49	113.8	502,636		8.4
2	22	5.0	14.2	22	7	42	42.9	251,185	13,225	3.5
Patient 4										
1	65	2.0	7.0	13	7	43	60.2	386,067		3.5
2	38	2.5	10.3	15	9	37	181.3	306,114		9.0
3§	42	5.0	43.0	45	19	5	139.1	125,317		3.5
3§	39	3.5	21.0	30	17	18	193.2	244,453	11,800	9.2
Patient 5										
1	54	6.5	13.3	27	10	35	100.6	359,198		3.8
2	57	1.5	7.0	17	8	53	166.2	591,278	13,978	3.1
Patient 6										
1	51	6.0	11.5	28	14	20	101.5	308,606		3.0
2	44	13.0	18.4	17	9	22	31.1	328,622	10,278	3.3
Patient 7										
1	55	5.0	10.5	21	12	46	50.1	472,861		3.7
2	41	1.0	6.5	12	2	21	197.8	385,305	11,002	3.8
Mean (±SD) values	45.0±14	4.8±3	13.9±9	24±12	10±5	30±15	132±67	357,336±109,042	11,547±1604	6.5±5

*The mean total beta-cell mass per transplant was based on the DNA content of the allografts (the DNA content of human islet cells is 6.0 pg per cell) and the percentage of insulin-positive cells in the allograft.[18]

†The islet equivalent (IE) is the standard unit used to report the volume of islets.

‡Values reflect the response of islet cells in vitro to a glucose challenge.

§Islets from two donors were used.

Table 7.1: Characteristics of the islet grafts

A mean packed-cell volume of3.5±1.3 ml was infused, and this did not change the portal pressure significantly (mean increase, 0.8 mm Hg; P=0.8). The results of tests of liver function 24 hours after transplantation were within the normal range. Doppler ultrasonography demonstrated no evidence of thrombus within the portal vein in any of the patients. The patients were hospitalized for a median of 2.3 days (range, 0.5 to 14.7), and three patients who underwent transplantation most recently (40 percent) were discharged within 24 hours after the procedure.

7.4.2 GLYCEMIC CONTROL AND SERUM C-PEPTIDE CONCENTRATIONS AFTER ISLET TRANSPLANTATION

Insulin requirements decreased in all patients after the first transplantation (Figure 7.2). Computer analysis of data from capillary glucose meters showed a marked improvement in glycemic control in all patients. Overall mean serum glucose concentrations decreased and the mean amplitude of glycemic excursions decreased significantly with sequential islet transplantation (Figure 7.2). The lability of glycemic control in a 24-hour period also decreased dramatically (Figure 7.3). All patients had normal glycosylated hemoglobin values after transplantation (Table 7.2). Serum C-peptide concentrations were undetectable in all patients before transplantation (less than 0.48 ng per milliliter after an overnight fast and in response to the mixed-meal test). Three months and six months after transplantation all patients had detectable serum C-peptide concentrations (P<0.001 by analysis of variance for the comparison with values before transplantation), and the concentrations did not decrease over time: at three months, the mean fasting value was 2.4 ±0.3 ng per milliliter (0.8 ±0.1 nmol per liter), and the mean value after a meal was 5.7 ±0.9 ng per milliliter (1.9 ±0.3 nmol per liter); at six months, the mean fasting value was 2.5 ±0.2 ng per milliliter (0.8 ±0.1 nmol per liter), and the mean value after a meal was 5.7 ±0.6 ng per milliliter (1.9 ±0.2 nmol per liter).

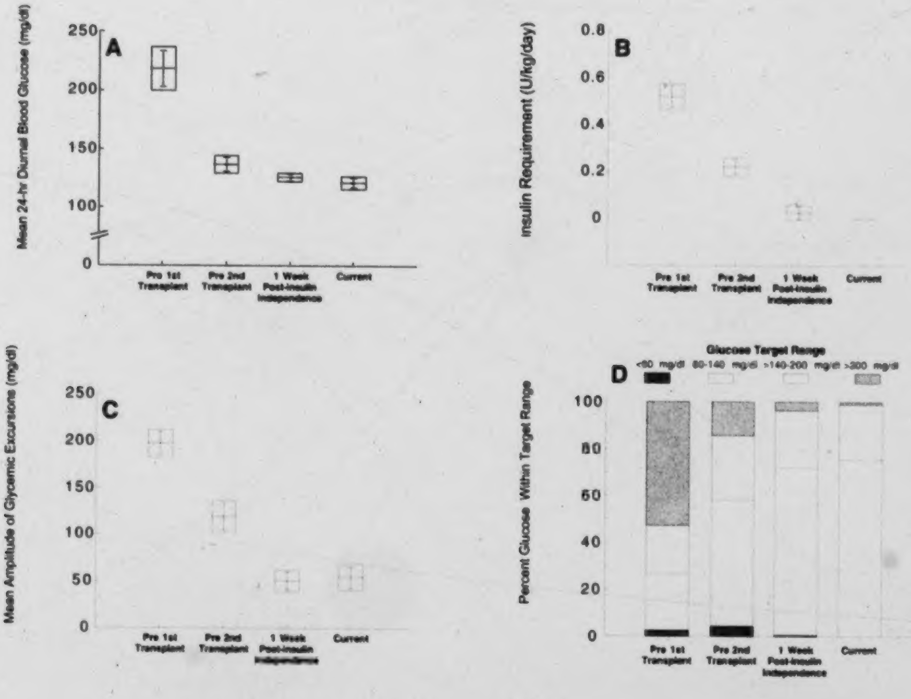

Figure 7.2: Mean 24-hour blood glucose at four sequential time intervals (3 days pre first transplant, 3 days pre second transplant, 1 week post insulin independence and most current data in follow-up).

Horizontal line represents mean value, error bars represent sem, and box represents 95 percent confidence intervals (P<0.001, analysis of variance). Mean ±sem and confidence intervals of daily insulin requirements expressed per recipient body weight (kg) over time (P<0.001, analysis of variance) Mean amplitude of glycemic excursions ±sem and confidence intervals over time (P<0.001, analysis of variance). Computer analysis of memory capillary glucose meter data. Mean percent of glucose values falling within each target range and statistical comparison are illustrated over time pre and post-transplant: <60mg/dl (<3.3mmol/l) (P=0.07), 60-140mg/dl (3.3-7.8mmol/l) (P<0.001), >140-200mg/dl (>7.8-11.1mmol/l) (P=0.93) and >200mg/dl (>11.1mmol/l) (P<0.001, analysis of variance). Conversion from mg/dl to mmol/l glucose, multiply by 0.05551

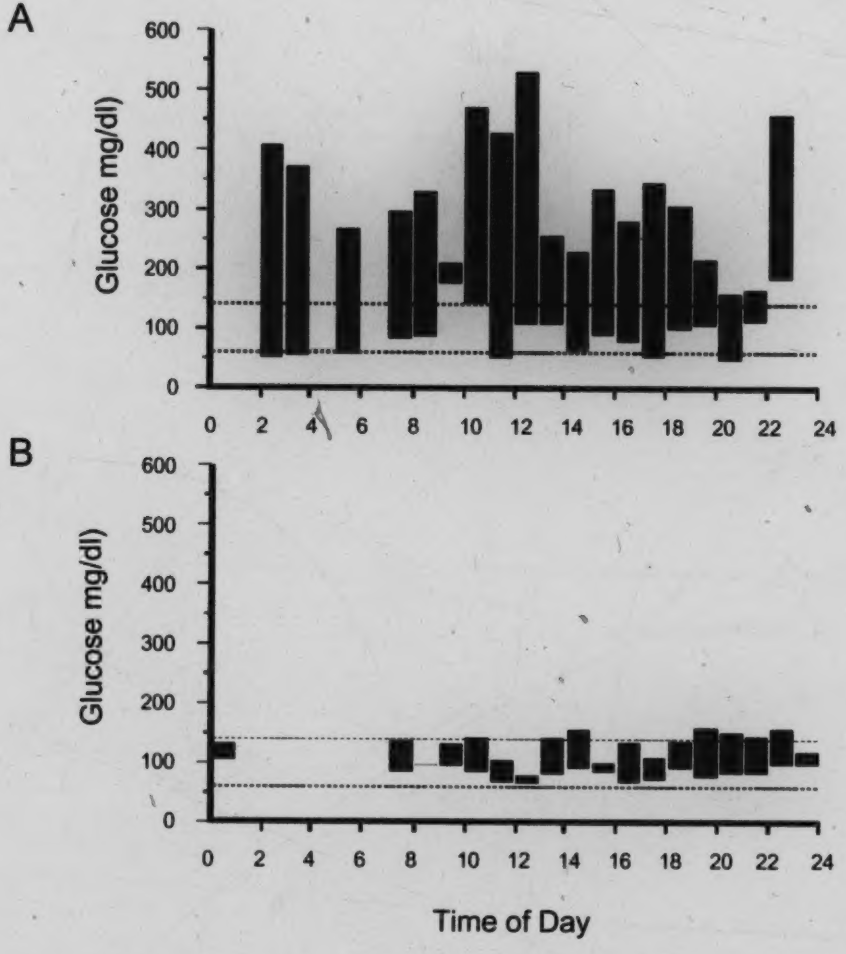

Figure 7.3: Fluctuation in glycemic profile documented over one-month pre-transplant (A) and after attainment of insulin-independence (B) in a representative islet recipient, (data expressed as median ±range).

The broken lines represent blood glucose levels of 60 and 140mg/dl (3.3mmol/l and 7.8mmol/l). Conversion from mg/dl to mmol/l glucose (or mean amplitude of glycemic excursions), multiply by 0.05551

7.4.3 AUTOANTIBODY ANALYSES

Serum was analyzed for anti-insulin antibody, islet-cell antibody 512, and glutamic acid decarboxylase antibody before and after transplantation(*19*). Mean serum anti-insulin antibody concentrations fell from 0.26 ±0.06 IU before transplantation to 0.07 ±0.03 IU after transplantation (P=0.04 by t-test); this change may represent a beneficial effect of systemic immunosuppression.

Serum glutamic acid decarboxylase antibody was undetectable before and after transplantation. One of four patients for whom data were available was positive for islet-cell antibody 512 before transplantation and remained so after transplantation.

7.4.4 ASSESSMENTS OF ORAL GLUCOSE TOLERANCE, MIXED-MEAL TOLERANCE, AND HOMEOSTASIS

The results of oral glucose-tolerance tests, completed after insulin independence had been achieved, indicated that none of the seven patients met current American Diabetes Association criteria for diabetes (Table 7.2) (*20*). However, in five patients, the response to the test at 120 minutes was impaired (glucose, 142 to 195 mg per deciliter [7.9 to 10.8 mmol per liter]), and two had fasting glucose concentrations that were at or above the upper limit of the normal range (110 mg per deciliter [6.1 mmol per liter]). We used the homeostatic model assessment(*18*) to estimate insulin sensitivity on the basis of paired fasting glucose and insulin data from the transplant recipients after they had achieved insulin independence and from normal subjects without diabetes. The values in the two groups did not differ significantly (103±14 percent among transplant recipients and 118±12 percent among control subjects, P=0.43).

TABLE 2. RESULTS OF ASSESSMENTS OF ORAL GLUCOSE TOLERANCE, MIXED MEAL TOLERANCE, GLYCOSYLATED HEMOGLOBIN VALUES, AND SERUM CREATININE AND LIPID CONCENTRATIONS BEFORE AND AFTER TRANSPLANTATION.*

PATIENT NO	DURATION OF FOLLOW-UP	GLUCOSE-TOLERANCE TEST†		MIXED-MEAL TEST‡			GLYCOSYLATED HEMOGLOBIN			CREATININE		CHOLESTEROL		TRIGLYCERIDE	
		GLUCOSE AFTER FASTING	GLUCOSE AT 120 MIN	C PEPTIDE AT 0 MIN	C PEPTIDE AT 90 MIN	GLUCOSE AT 90 MIN	BEFORE TRANSPLANTATION	AT 3 MO	AT 6 MO	BEFORE TRANSPLANTATION	AT FOLLOW-UP	BEFORE TRANSPLANTATION	AT FOLLOW-UP	BEFORE TRANSPLANTATION	AT FOLLOW-UP
	mo	mg/dl		ng/ml		mg/dl		percent				mg/dl			
1	14.9	103	130	3.6	5.7	150	7.2	6.2	5.7	1.9§	2.3	149	155	78	77
2	12.8	97	195	1.4	3.0	157	8.1	5.6	5.9	0.8	0.8	210	208	134	117
3	12.3	108	142	1.6	3.6	182	9.0	5.6	5.8	1.1	1.0	210	149	86	124
4	11.9	88	139	2.3	5.1	83	8.6	5.7	5.5	1.5	1.4	166	189	80	137
5	9.0	110	150	2.8	4.8	106	9.0	5.5	5.8	0.9	0.8	247	168	163	93
6	5.5	113	157	2.0	4.8	153	8.5	5.5	—	1.8	1.8	180	260	132	167
7	4.4	92	175	—	—	—	8.6	5.8	—	1.0	0.7	188	240	66	120
Mean ± SD		101.7±10	155.2±23	2.3±0.8	4.5±1.0	138.4±37	8.4±0.6	5.7±0.2¶	5.7±0.2¶	1.3±0.4	1.2±0.6	193±33	196±43	105±37	119±29

*Values were determined after insulin independence had been achieved. To convert values for glucose to millimoles per liter, multiply by 0.0555. To convert values for creatinine to micromoles per liter, multiply by 88.4. To convert values for C peptide to nanomoles per liter, multiply by 0.33. To convert values for cholesterol to millimoles per liter, multiply by 0.026. To convert values for triglyceride to millimoles per liter, multiply by 0.011.

†A total of 75 g of glucose in 300 ml of solution was given over a one-minute period after an overnight fast.

‡A total of 300 ml of a high-protein drink (Ensure) was given over a one-minute period.

§This patient had preexisting renal dysfunction, with a glomerular filtration rate of 46 ml per minute per square meter of body surface area on radionuclide assay.

¶P<0.001 for the comparison with the value before transplantation by analysis of variance.

Table 7.2: Oral glucose tolerance, mixed meal, creatinine and lipid data.

7.4.5 TRANSPLANTATION-RELATED COMPLICATIONS

None of the patients have had cytomegalovirus infection, despite the fact that four were seronegative for the virus before transplantation and received an allograft from a seropositive donor. In the first 2 of the 15 procedures, moderate bleeding occurred at the site of the transhepatic puncture and required transfusion. This complication was subsequently avoided by injecting a Gelfoam plug through the catheter and by reducing the intraportal dose of heparin from 5000 to 500 U. All patients had minor, superficial ulcerations of the buccal mucosa that resolved after the dose of sirolimus was reduced and the capsule formulation of sirolimus was substituted for the liquid form. None of the patients had sirolimus-related cytopenia.

After transplantation, there were no significant increases in lipid concentrations and no patient required lipid-lowering therapy (Table 7.2). There were no significant changes in serum concentrations of creatinine (P=0.92), cholesterol (P=0.90), or triglycerides (P=0.46) during follow-up (Table 7.2). As of this writing, there has been insufficient follow-up for us to perform a prospective evaluation of secondary diabetic complications.

7.5 DISCUSSION

We found that in patients with type 1 diabetes the use of a glucocorticoid-free immunosuppressive protocol in conjunction with islet transplantation quickly resulted in sustained freedom from the need for exogenous insulin. Our results represent an improvement in outcome as compared with previous reports(*1*). Transplantation of an initial, sub-optimal islet mass halted the episodes of severe hypoglycemia in our patients. Sirolimus, low-dose tacrolimus, and daclizumab provided effective immunosuppression without diabetogenic or toxic effects. Indeed, there were no clinically evident episodes of graft rejection, and this combination appears to be effective in preventing autoimmune recurrence of diabetes. A recent review of the potential barriers to insulin independence after islet transplantation identified several factors(*21*). The number of beta cells may be inadequate owing to insufficient engraftment of islets and immediate cellular loss through apoptosis and other non immune-mediated inflammatory pathways(*22, 23*). The graft may be rejected as a result of ineffective immunosuppression of both alloimmune and autoimmune pathways(*24*) This event is hard to identify initially, given the lack of tools available for the early diagnosis of rejection(*25*). The high metabolic demand on the islets that results from preexisting insulin resistance in most patients who undergo combined islet and kidney transplantation is aggravated by the use of diabetogenic immunosuppressant agents(*4, 26*). We addressed each of these key factors by transplanting an adequate number of viable, well-characterized islets, which had been prepared in xenoprotein-free medium, and minimizing the duration of cold ischemia. Nonspecific coating of islets by a xenoprotein could theoretically target such cells for immediate destruction. The immunosuppressive regimen that we used protected against alloimmune and autoimmune reactivity. The use of a glucocorticoid-free protocol that included low-dose tacrolimus and daclizumab further minimized the possibility of damaging beta cells and increasing insulin resistance. Interest in the use of sirolimus increased when its molecular structure was found to be similar to that of tacrolimus(*27*). Sirolimus-based trials of kidney transplantation reported a substantial reduction in the rate of acute rejection with minimal nephrotoxicity(*28, 29*). Preclinical studies of the use of sirolimus with islet transplantation reported prolonged allograft survival and enhanced autograft function(*30, 31*). In vitro studies suggested that sirolimus and tacrolimus could not be used in combination, since both drugs bind to the same cytosolic binding proteins (FKBP-12 and FKBP-25)(*32*). This interaction does not occur when the two are used in vivo, and indeed, there is a strong synergistic potentiation of efficacy(*33, 34*). The combination of sirolimus, low-dose tacrolimus, and glucocorticoids in liver, kidney, and pancreas transplantation has been associated with extremely low rates of rejection(*35*).

To avoid the diabetogenic effect of glucocorticoids in islet transplantation, we replaced them with daclizumab. This monoclonal antibody against the interleukin-2 receptor has been shown to be safe and effective in renal transplantation, and its use lowered the rates of rejection(*36*). Daclizumab therapy is given over a 10-week period, thus allowing an extended period for a supplemental islet-transplant procedure. The combined glucocorticoid-free strategy of tacrolimus, sirolimus, and daclizumab therapy prevents activation of the immune cascade by inhibiting T-cell activation, the production of interleukin-2 and other cytokines, binding of the interleukin-2 receptor to its ligand, and the clonal expansion of lymphocytes(*37*).

Our findings show that an infusion of islets from a single donor (a mean of 389,016 ± 73,769 islet equivalents in the first transplant) did not result in insulin independence. Since glucocorticoids were not used and thus did not exert any adverse effects on islet function, other factors must be involved. The quantity of islets required to achieve insulin independence is approximately double that reported previously(*1*). Recently, one center achieved insulin independence in 14.3 percent of patients after the transplantation of islets from a single donor, but insulin was not withdrawn until a mean of 10.6 months after transplantation(*38*). In our study the need for more than one donor pancreas per recipient may be interpreted as a drawback, given the shortage of donors. At present, however, less than one third of available cadaveric pancreases are actually transplanted (United Network for Organ Sharing Registry: unpublished data). In patients with type 1 diabetes, glycemic control can also be achieved with intensive insulin therapy and pancreatic transplantation. Intensive insulin therapy does not normalize glycosylated hemoglobin values and may cause severe hypoglycemia(*39*). Pancreatic transplantation provides excellent glycemic control, and although the outcome of the procedure has improved dramatically over the past decade, it remains an invasive procedure with a substantial risk of morbidity(*40*). Our findings indicate that islet transplantation alone is associated with a minimal risk and results in good metabolic control, with normalization of glycosylated hemoglobin values and sustained freedom from the need for exogenous insulin.

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CHAPTER 8:

THE "EDMONTON PROTOCOL" – UPDATED RESULTS

8.1 THE "EDMONTON PROTOCOL" – UPDATED RESULTS

As discussed in detail in Chapter 6, a new protocol was introduced in 1999 for clinical islet transplantation. The major changes to the previous protocol involved:

1. Potent immunosuppression without glucocorticoid exposure, using daclizumab induction with maintenance sirolimus and low-dose tacrolimus.
2. Focus on a new group of patients without renal failure, for islet-alone transplantation
3. Use of "double-donor" transplants to provide an adequate therapeutic islet mass to sustain independence from insulin.
4. Pancreas perfusion, Liberase digestion, continuous gradient commercial Ficoll
5. Immediate transplantation of freshly isolated islets, without culture or use of cryopreserved tissue.
6. Elimination of xenoproteins used previously during islet isolation (fetal calf serum) with more compatible human albumin solutions.
7. A multidisciplinary team approach to the design and rapid implementation of a new protocol.

8.2 DEMOGRAPHICS

A total of 15 consecutive patients with type 1 diabetes have now been treated in Edmonton using this protocol. One additional patient has received the initial transplant, and is currently waiting for a second islet infusion; data from this patient has therefore not been included herein. Two further patients have recently undergone transplantation under a modified "single-donor" protocol with the addition of anti-TNF alpha (infliximab). One patient is completely insulin independent under the single-donor protocol, and the second patient is using only 4-6 units of insulin per day. While these early results are encouraging, longer follow-up in an expanded series of patients will be needed to define benefit of anti-TNF alpha blockade in a single-donor islet transplant setting.

The median recipient age is 40 years (range 29-53), and the median duration of diabetes is 31 years in this group. The male: female ratio is 1.8:1. All patients were considered for islet-alone transplantation after confirming that stimulation pre-transplant C-peptide levels were undetectable. All patients had chronic complications of diabetes, including metabolic lability in 86%, microalbuminuria in 64%, retinopathy in 50%, neuropathy in 29% and vasculopathy in 7% (Table 8.1).

Demographics

[redacted]				[redacted]				
1	45	35	neg	+	+	+	+	+
2	52	50	neg	+	-	+	+	+
3	29	18	neg	-	-	+	+	+
4	53	39	neg	+	-	+	+	+
5	43	32	neg	+	-	-	+	+
6	34	22	neg	-	+	-	+	+
7	40	36	neg	-	-	-	+	+
8	31	29	neg	+	-	-	+	+
9	39	10	neg	-	-	+	+	+
10	30	13	neg	-	-	-	-	+
11	30	14	neg	-	-	-	+	+
12	40	34	neg	+	+	-	+	-
13	41	34	neg	-	-	-	-	+
14	36	17	neg	-	-	-	+	-
15	43	31	neg	+	+	-	+	+

[redacted]

Table 8.1: Demographics updated to 15 consecutive patients

These patients have generally tolerated the immunosuppressive protocol without major side effects, and as can be seen in Figure 8.1, they do not have the steroid "moon facies" that transplant patients traditionally would have had since they have not received any glucocorticoid therapy.

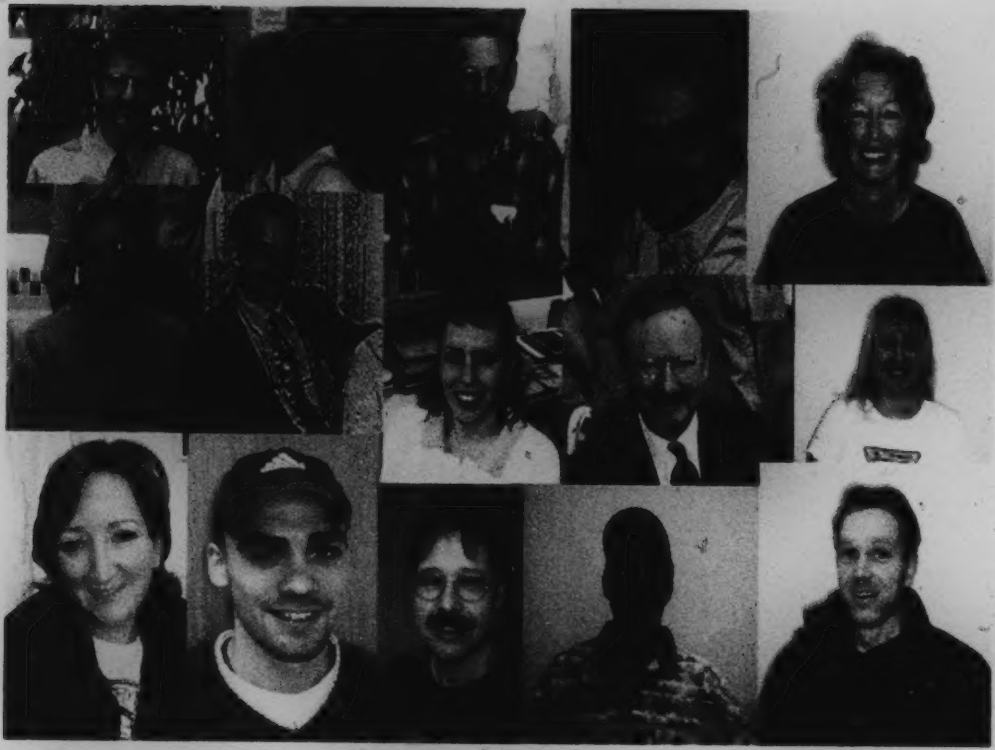
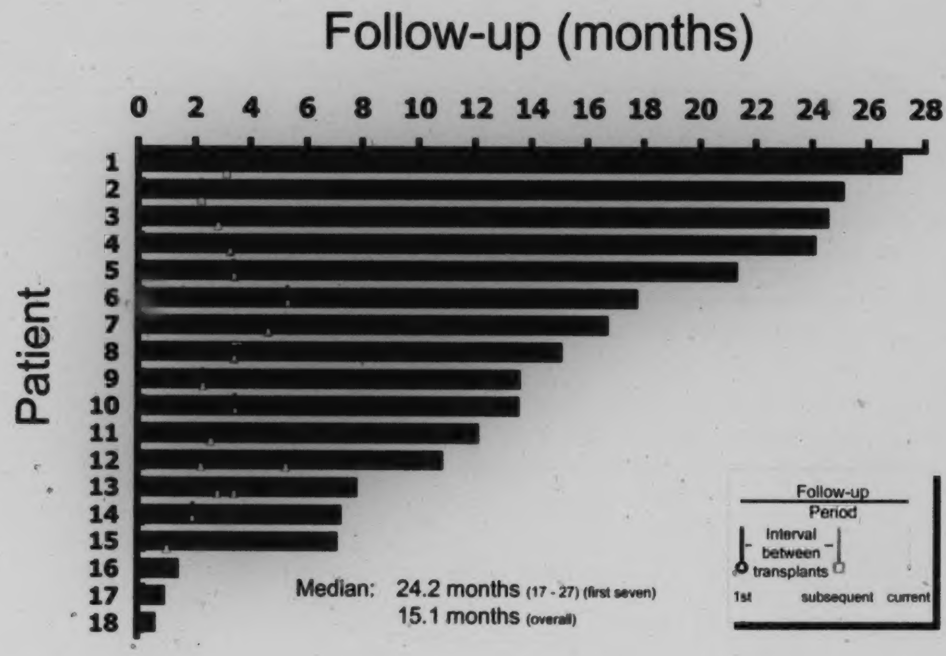


Figure 8.1: The patients have tolerated their immunosuppression relatively well, and as shown there are no steroid "moon facies" since glucocorticoids were not given.

8.3 FOLLOW-UP

The median follow-up of the initial cohort of seven patients is currently 24.2 months (range 17-27). At the present time, the first four patients are over two years following transplantation and remain insulin free. Of the first seven patients reported by our group, 6/7 still remain insulin-free currently in long-term follow up. One patient is using small amounts of nocturnal insulin to optimize control, and it is planned that this patient will receive a further supplemental transplant from a fresh “sub-therapeutic” islet preparation that could not be used to treat a new patient because of insufficient mass. The overall follow-up for the 15 consecutive patients is now 15.1 months (Figure 8.2).



Median: 24.2 months (17 - 27) (first seven)
15.1 months (overall)

Figure 8.2: Follow-up, and timing of subsequent transplant in 16 consecutive patients (shown in blue). Two further patients (shown in red) have undergone islet transplantation under a new “single donor” infliximab-based protocol.

The median delay between first and subsequent transplant was 29 days (range 0-71 days), and was dependent upon the availability of a fresh islet isolation of adequate yield. In general the initial transplants were ABO identical, and the subsequent transplants were ABO-compatible to minimize a need for a protracted course of daclizumab.

8.4 DONOR REQUIREMENTS AND ISOLATION DATA

Islets were prepared from "double-donors" in the majority of cases (12/16). Three recipients received islets from three donors, and one very unusual case received islets from four donors (because of excessive recipient weight combined with a technical failure in purification runs) (Figure 8.3).

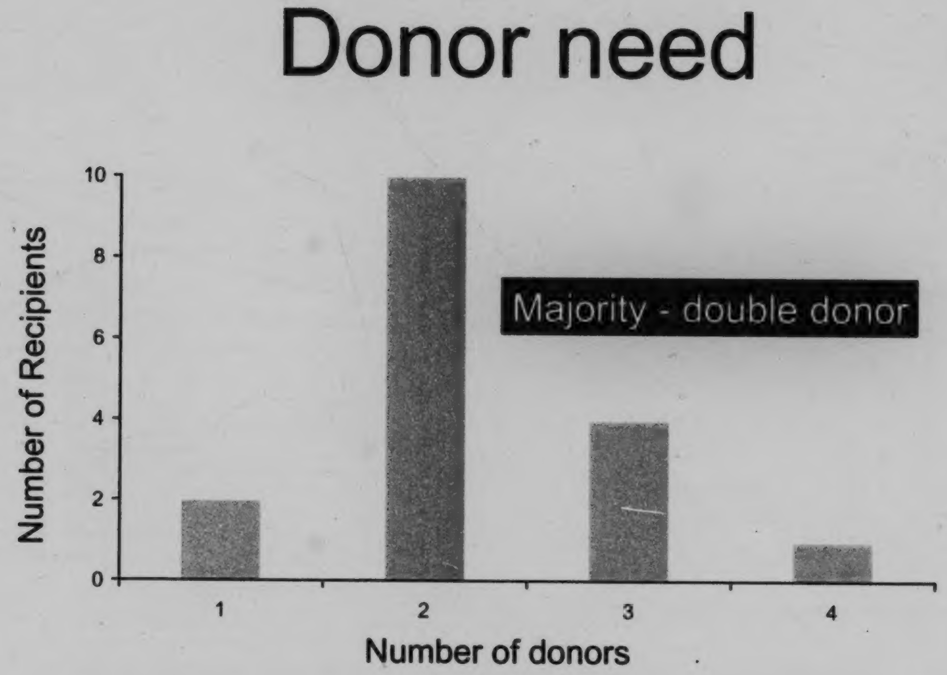
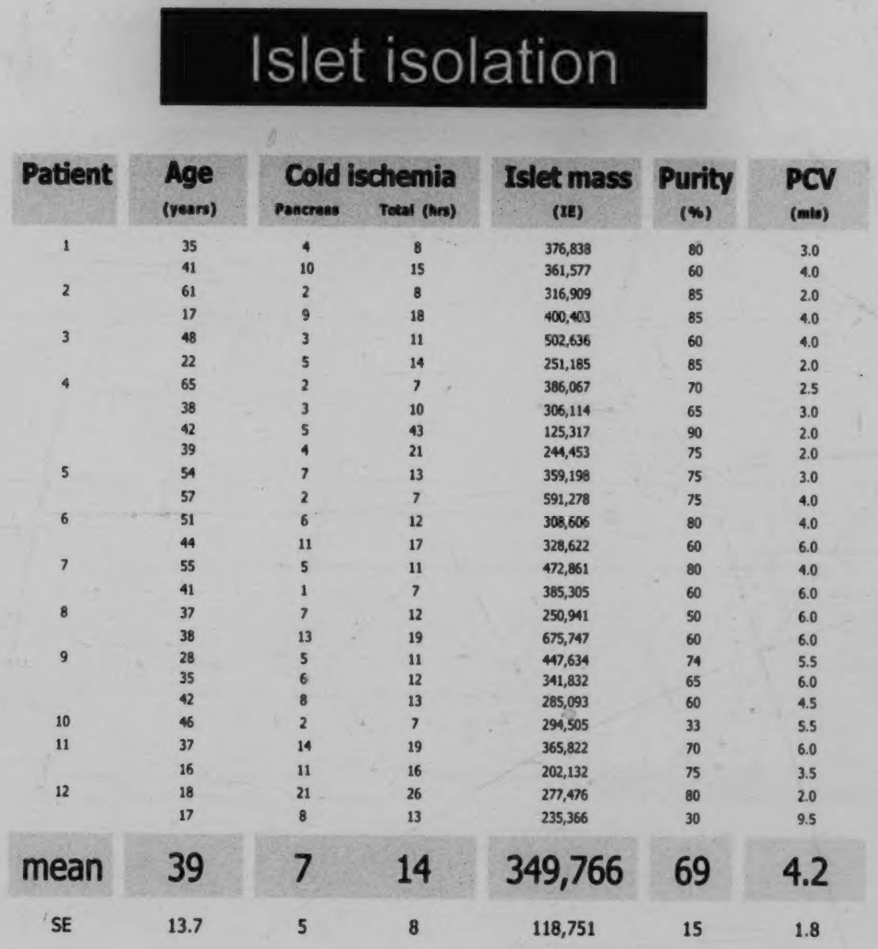


Figure 8.3: Number of donors required versus number of recipients. The majority of recipients received "double donor" transplants. (Orange – Edmonton protocol; pink – infliximab protocol).

(Note: With limited follow-up in the infliximab protocol, one patient is currently insulin independent after single donor islet transplantation, and the second patient is using between 4-6U insulin per day . Further interpretation must await more detailed follow-up in an expanded patient series).

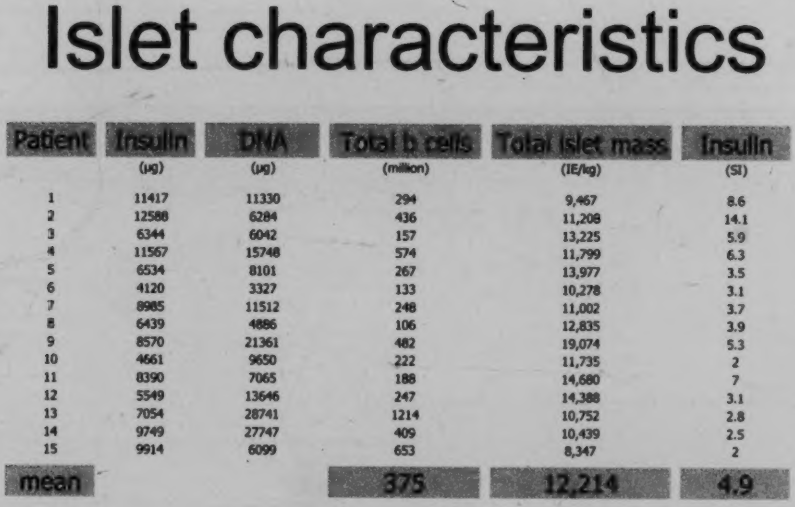
The mean donor age was 39 years (range 17-71); mean cold ischemia (aortic cross-clamp to start of islet processing was 7 hours. The mean islet infusion mass was 349,766 IE (data from the first 12 patients); islet purity was estimated at 69%, and the mean packed cell volume after islet purification was 4.2cc (Table 8.2). The mean islet mass was 12,214 islet equivalents per kg (range 8,347-19,074, based on the recipient body weight). The mean calculated beta cell content 276,000,000 cells per transplant (Table 8.3). Islets were functional in vitro, with membrane dye exclusion confirming viability in over 95% of cells and insulin release in static incubation in high glucose of 6 times the basal output. The graft endocrine composition consisted of 24% beta cells, 10% alpha cells, 4% delta cells and 23% CK19 positive ductal cells, when assessed by immuno-histochemistry. The high content of ductal elements may be explained by the Ficoll purification process – since ductal elements have similar density to islets and are therefore enriched by purification. The high content of ductal elements might have important future bearing on long-term islet function, since they have the potential capacity for islet neogenesis(*1*).



Islet isolation

Patient	Age	Cold ischemia		Islet mass	Purity	PCV
	(years)	Pancreas	Total (hrs)	(IE)	(%)	(mls)
1	35	4	8	376,838	80	3.0
	41	10	15	361,577	60	4.0
2	61	2	8	316,909	85	2.0
	17	9	18	400,403	85	4.0
3	48	3	11	502,636	60	4.0
	22	5	14	251,185	85	2.0
4	65	2	7	386,067	70	2.5
	38	3	10	306,114	65	3.0
	42	5	43	125,317	90	2.0
	39	4	21	244,453	75	2.0
5	54	7	13	359,198	75	3.0
	57	2	7	591,278	75	4.0
6	51	6	12	308,606	80	4.0
	44	11	17	328,622	60	6.0
7	55	5	11	472,861	80	4.0
	41	1	7	385,305	60	6.0
8	37	7	12	250,941	50	6.0
	38	13	19	675,747	60	6.0
9	28	5	11	447,634	74	5.5
	35	6	12	341,832	65	6.0
	42	8	13	285,093	60	4.5
10	46	2	7	294,505	33	5.5
11	37	14	19	365,822	70	6.0
	16	11	16	202,132	75	3.5
12	18	21	26	277,476	80	2.0
	17	8	13	235,366	30	9.5
mean	**39**	**7**	**14**	**349,766**	**69**	**4.2**
SE	13.7	5	8	118,751	15	1.8

Table 8.2: Islet isolation parameters.
(Data generously provided by Dr Jonathan Lakey)



Islet characteristics

Patient	Insulin (µg)	DNA (µg)	Total b cells (million)	Total islet mass (IE/kg)	Insulin (SI)
1	11417	11330	294	9,467	8.6
2	12588	6284	436	11,208	14.1
3	6344	6042	157	13,225	5.9
4	11567	15748	574	11,799	6.3
5	6534	8101	267	13,977	3.5
6	4120	3327	133	10,278	3.1
7	8985	11512	248	11,002	3.7
8	6439	4886	106	12,835	3.9
9	8570	21361	482	19,074	5.3
10	4661	9650	222	11,735	2
11	8390	7065	188	14,680	7
12	5549	13646	247	14,388	3.1
13	7054	28741	1214	10,752	2.8
14	9749	27747	409	10,439	2.5
15	9914	6099	653	8,347	2
mean			375	12,214	4.9

Table 8.3: Islet graft characteristics
(Data kindly provided by Dr Gregory Korbutt)

Figure 8.4: Use of fluoroscopic guidance by an expert interventional radiologist to gain access to the portal vein.

8.5 PERCUTANOUS ACCESS TO THE PORTAL VEIN

Islet transplantation was completed by percutaneous transhepatic portal vein access under fluoroscopic guidance in the awake patient in all cases, allowing over 90% of patients to be discharged from hospital between 12 and 24 hours post transplant. The combination of ultrasound and fluoroscopic guidance, together with increased familiarity and expertise of the procedure by the interventional radiology team, now permits rapid access to the main portal vein – often within 15-30 minutes (Figure 8.4). Use of a modified 4 French Cook catheter (inner lumen 900µm), now termed the "O'Kelly islet infusion catheter," has reduced the procedural risk of bleeding considerably, and has made the use of a thrombostatic plug redundant.

Figure 8.5: Portal angiogram demonstrating placement of the catheter within the main vein.

(Note in this case the catheter tip is initially in the superior mesenteric vein, and was retracted slightly prior to islet embolization). Exposure to contrast media is limited once correct placement is confirmed, to avoid potential toxicity of contrast media to the islets.

Currently 30 iU/kg of heparin is mixed with the initial islet preparation, and the patient is then maintained on low molecular weight heparin (Lovenox 30mg sc) and low dose aspirin (80mg) for seven days post transplant, both to reduce the risk of portal vein thrombosis and also to reduce islet loss related to platelet activation(*2*). There were no significant changes in portal pressure comparing pre-infusion values to completion readings, although transient mild elevations (5-10mmHg) were encountered in patients receiving more than 7ml of packed tissue volume, which returned close to baseline on completion of the infusion.

8.6 INSULIN INDEPENDENCE AND METABOLIC CONTROL

This original series has now been extended to 15 patients, all of whom were able to achieve insulin independence for longer than one month. A further patient (#16) awaits a second transplant at the current time. All patients had complete correction of severe hypoglycemic reactions, leading to a marked improvement in overall quality of life (see below). All patients continue to have persistent and detectible levels of C-peptide. Ten of 15 patients remain completely free of insulin currently, and five are using small amounts of insulin (ranging between 1/5th and 3/4 of their pre-transplant dose), for varying reasons: One patient received a marginal islet infusion mass; two clearly became insulin resistant (HOMA 48 vs. mean 112 in remaining patients) possibly as a result of tacrolimus therapy; one lost partial graft function related to a peripheral segmental thrombosis of the right portal vein (technical in origin, since islets were infused peripherally rather than centrally in this case, and a liquefied thrombostatic preparation was embolized rather than a solid plug in this exceptional case); the fifth patient may have lost partial graft function related to low sirolimus levels.

The patients with insulin resistance have not corrected with combination biguanide and thiazolidinedione therapy. Even the five patients using modest insulin have much more stable glycemic control than they had pre-transplant, and in particular have not had recurrent hypoglycemic comas, and should therefore not be regarded as failures. It is anticipated that these patients will achieve insulin independence after supplemental islet infusions with 'sub-therapeutic' grafts that cannot be used in new patients.

The overall mean 24-hour glucose values have been significantly improved by islet transplantation ($p < 0.001$, ANOVA), and remain so over time, as illustrated in Figure 8.6. Hypoglycemic comas or reaction events have been avoided entirely in all patients, and none have required third party assistance or hospitalization related to hypoglycemia post transplant, in marked contrast to their pre-transplant state. Insulin-free patients did not experience glucose values less than 3.5mmol/L. In two of five patients using insulin, occasional glucose values have been detected below 3.5 mmol/L, as shown in Figure 8.6 at 6 and 12 months post-transplant, but these have not been associated with hypoglycemic reaction events. Therefore even the five patients using between 1/5th and 3/4 of their pre-transplant insulin requirements demonstrate glycemic control that is considerably improved compared to before transplantation.

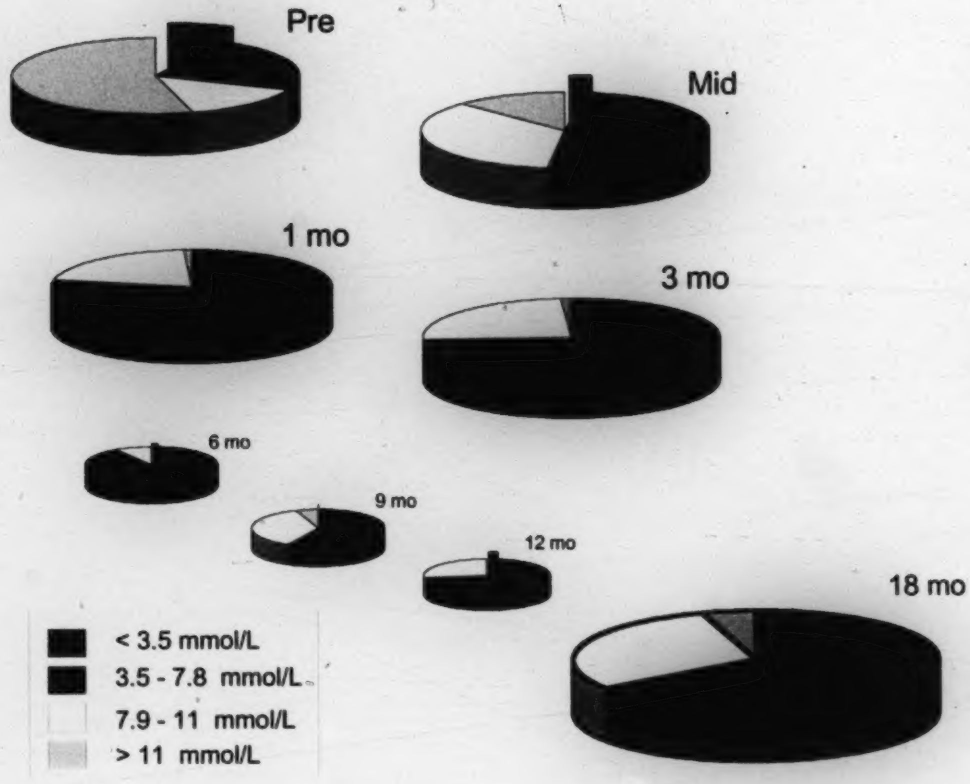


Figure 8.6: Mean glycemic control (as measured by memory glucose meters and analyzed using precision Link 2.0 software) is significantly improved, compared to values pre-transplant, and importantly hypoglycemic events (glucose < 3.5 mmol/L, red) are prevented in insulin-free patients.

Glycated hemoglobin has been corrected by islet transplantation in most cases (normal < 6.1%, Figure 8.7), which could not be achieved by intensive insulin therapy in the DCCT trials(*3*).

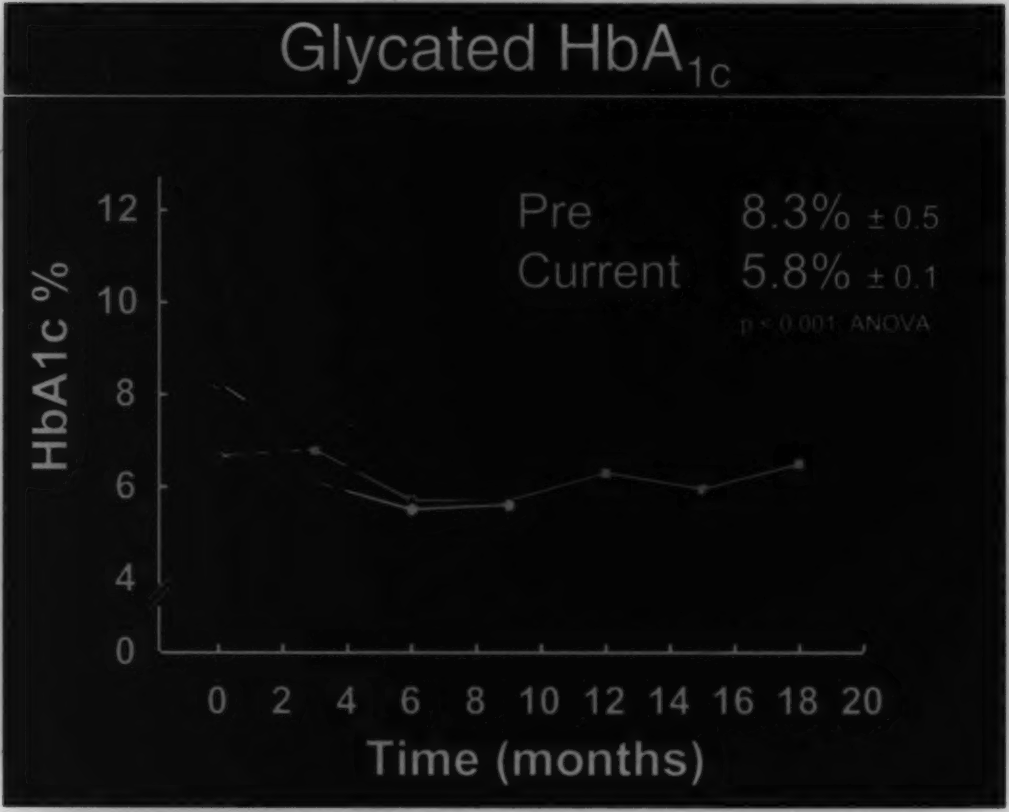


Figure 8.7: Improvement in glycated hemoglobin after islet transplantation.

Using a memory glucose meter, 24-hour glucose profiles are corrected to near-normal following islet transplantation, with improved control of fluctuations in glycemic excursion (Figure 8.8).

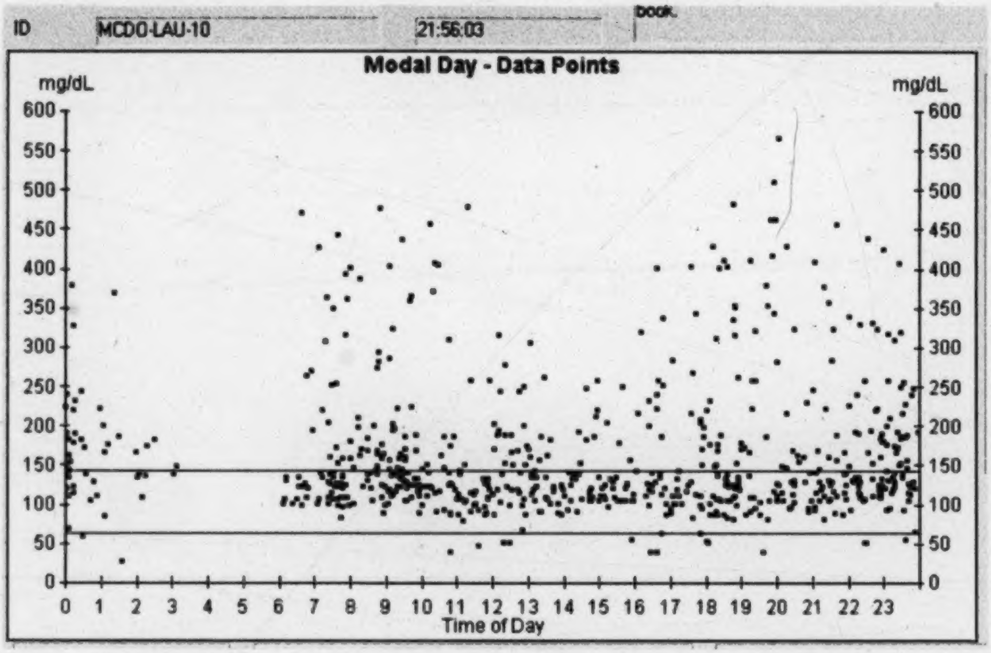


Figure 8.8 (a): 24-hour excursion in glycemic control PRE-TRANSPLANT for patient LM

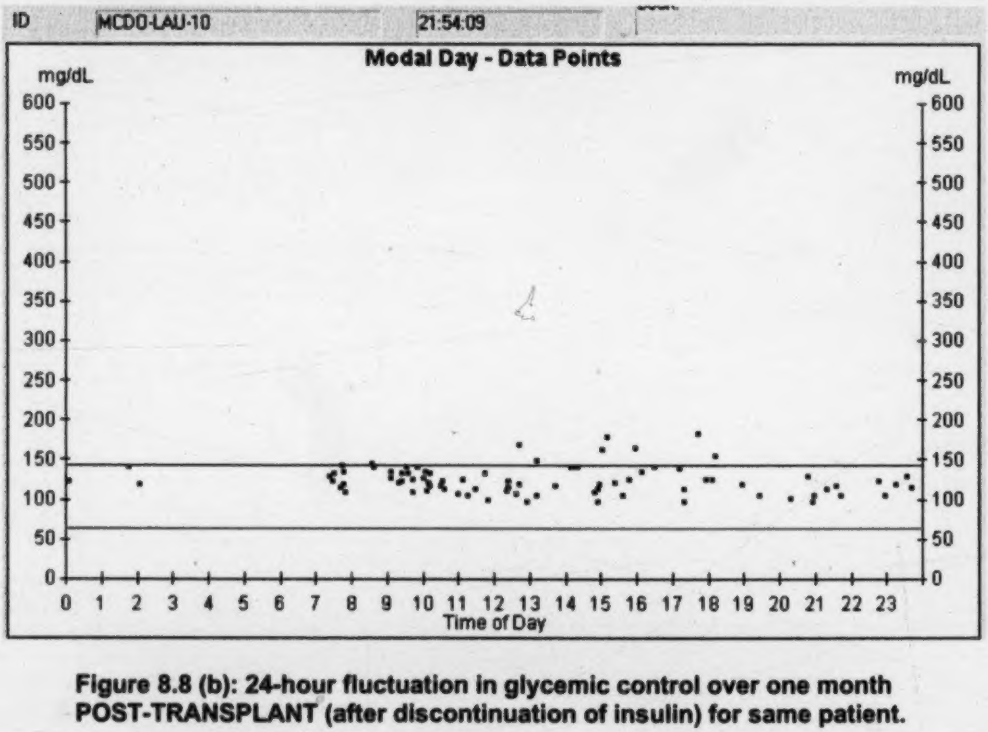


Figure 8.8 (b): 24-hour fluctuation in glycemic control over one month POST-TRANSPLANT (after discontinuation of insulin) for same patient.

There was a tight relationship between transplanted islet mass and chance of attainment of insulin independence (Figure 8.9) – with insulin requirements falling to zero once a minimum of 9,000 – 10,000 IE/kg islet mass had been infused (Figure 8.10). The islet infusion mass required to attain insulin independence was substantially higher (66% more) than previously recommended as a minimal islet mass according to the Islet Transplant Registry(*4*).

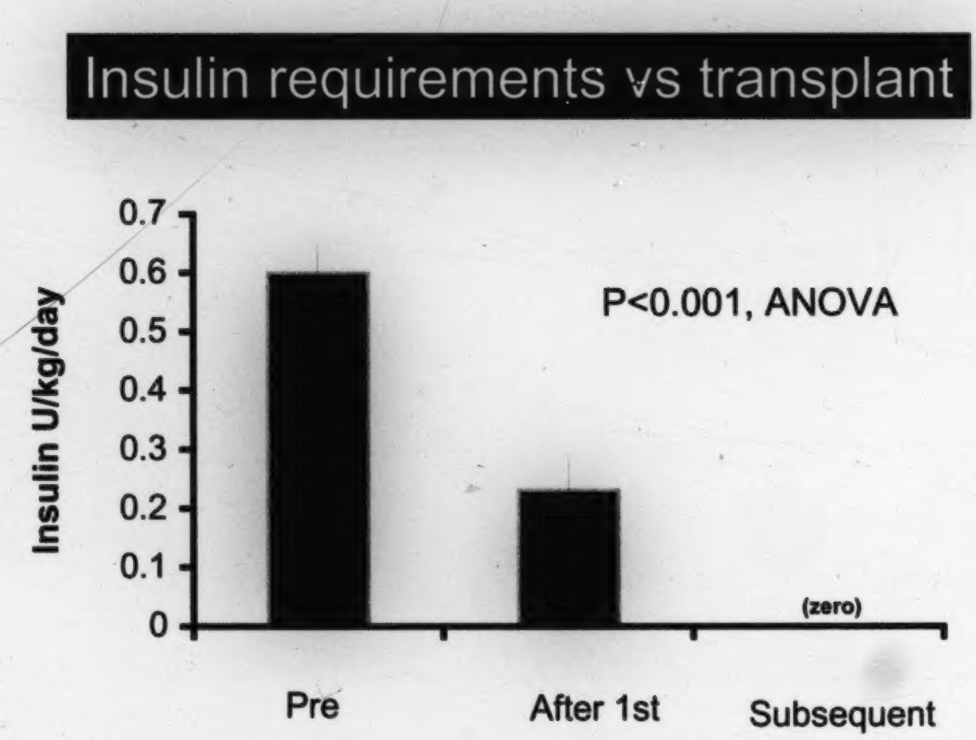


Figure 8.9: Relationship between insulin requirement (units per kg per day) pre-transplant vs. after first transplant vs. after second (or subsequent) transplant (mean ± SEM).

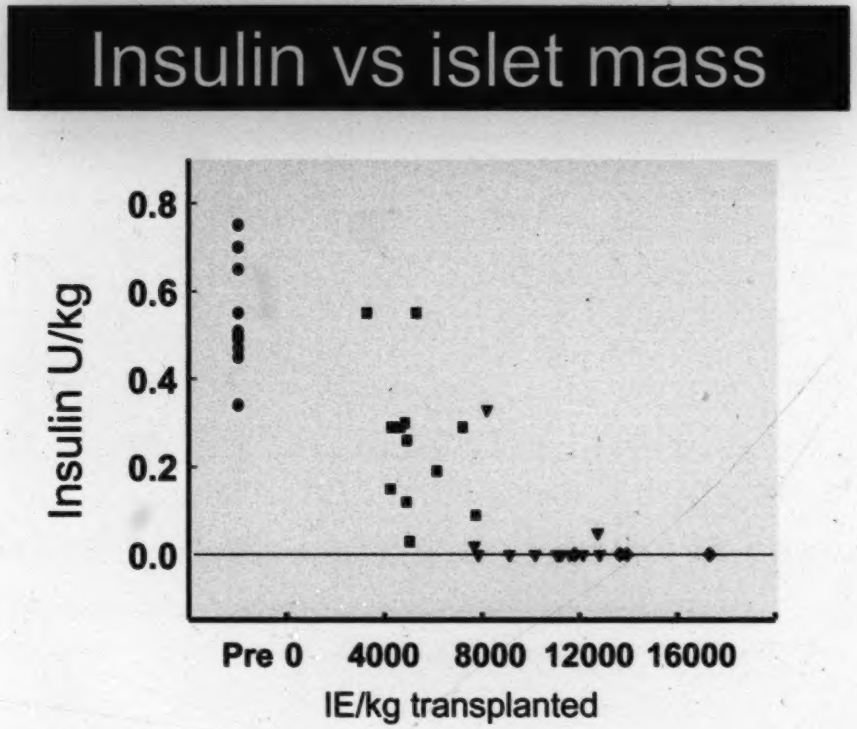
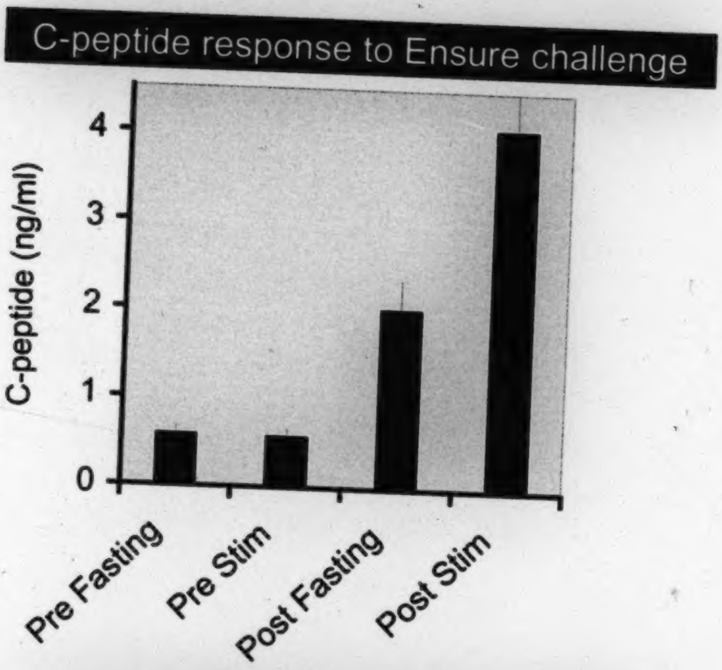


Figure 8.10: Relationship between total daily insulin requirement and islet implant mass – insulin independence is not achieved unless a minimal islet mass of 9,000 – 10,000 IE/kg has been transplanted.

It has been estimated that the average adult human pancreas contains between 1 and 14.8 million islets of mean diameter 157 µm(*5, 6*). Others claim that the upper limit of islet mass in a normal adult pancreas is approximately 2 million islets. The average islet yield in successful isolations in the current series was 349,766 IE. Therefore the estimated islet recover was between 2.4% (17%) and 35% of the potential mass. The current techniques used for islet isolation remain crude, and there is clearly enormous residual potential for improvements in the science of islet isolation. The mean islet infusion mass was 830,000 IE in this series. Thus, between 5.6% (41%) and 83%of the normal islet complement was transplanted.



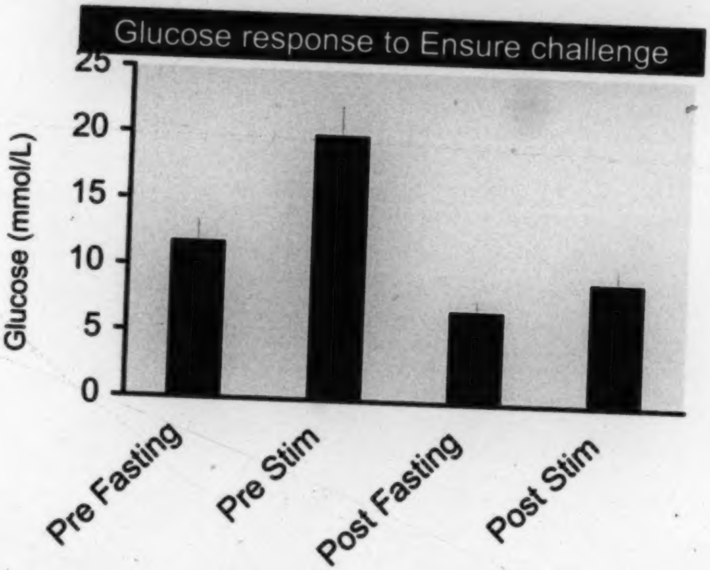
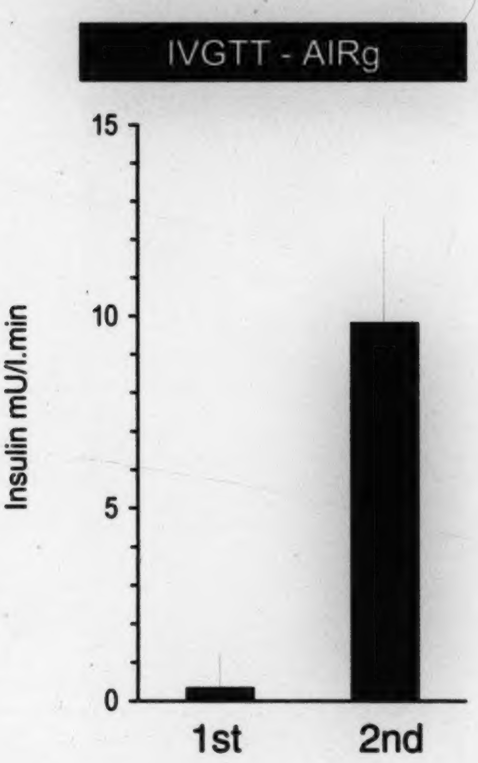


Figure 8.11: C-peptide and glucose response to Ensure challenge (fasting and 90 min stimulated) – pre-transplant (Pre) vs. most current (Post) data (mean ± sem) for 15 patients (C-peptide ng/ml).

The metabolic data defined below clearly demonstrates that despite infusion of a substantial islet mass, and in the absence of glucocorticoids, these patients have approximately one fifth of normal functional insulin reserve(*7*). It is evident that a significant proportion of islets fail to engraft based on the above estimates – perhaps only between 24% and 48% of islets survive after infusion(*2*).

Based on data from intravenous glucose tolerance tests performed after the first and subsequent transplant, Ryan *et al* reported that the acute insulin response to glucose (AIRg) increased only fractionally after the first transplant, but the response to the subsequent graft was considerably higher (Figure 8.12) (*7*).

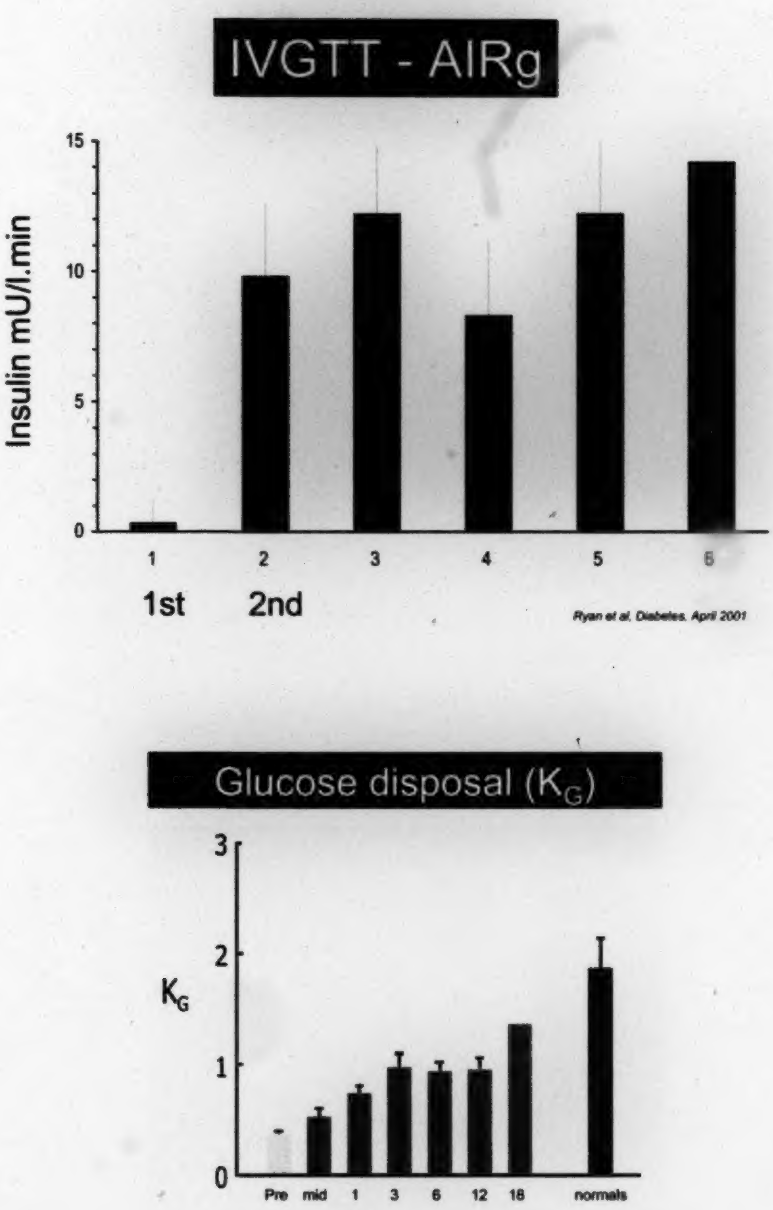


Ryan et al, Diabetes 50:710-719, 2001

Figure 8.12: Acute insulin response to glucose after first and subsequent transplant (based on IVGTT)

It is not entirely clear why the AIRg response to the subsequent graft is so much more potent, when a similar mean islet mass (approximately 350,000 IE) were infused on each occasion. Possible explanations include: a) some form of tolerance (either to auto or allo-antigens) induced by the first graft (there is no evidence to substantiate this), b) improved islet survival related to a more favorable and stable metabolic environment for the second graft (reduced islet metabolic stress and 'burn-out'), c) the second graft is implanted under more stable and more prolonged immunosuppression, reducing potential immune-mediated graft loss. Other more plausible explanations include d) the differential response is a reflection of an artefact of neovascularization – a process which is more complete in the initial graft at the time of delayed re-challenge, whereby the islets may respond more rapidly to an acute glucose challenge. An alternative explanation could be e) the fact that with additional islet reserve, the beta cells are less degranulated at the time of the glucose challenge, and therefore can respond with an augmented insulin response.

An important observation based on serial IVGTT's over time is that the mean AIRg remains stable and does not diminish (Figure 8.12), providing encouraging support that the islet engraftment mass does not degrade significantly over time. This fact is further supported by the glucose decay data (K_G) in the IVGTT's, which have also remained stable over time (Figure 8.12). When compared to a concurrent cohort of healthy non-diabetic controls, the islet recipients have on average one fifth of the normal insulin reserve(7). As a consequence of this, the majority of patients have impaired glucose tolerance, and three patients that were free of insulin under normal conditions have required temporary insulin at times of stress from intercurrent viral illness.



Ryan et al. Diabetes, April 2001

Figure 8.13: Mean acute response to glucose (AIRg) and glucose decay (K_G) remains stable over time – but AIRg is only 1/5th of normal, compared to healthy non-diabetic controls ***(mean ± SEM).***

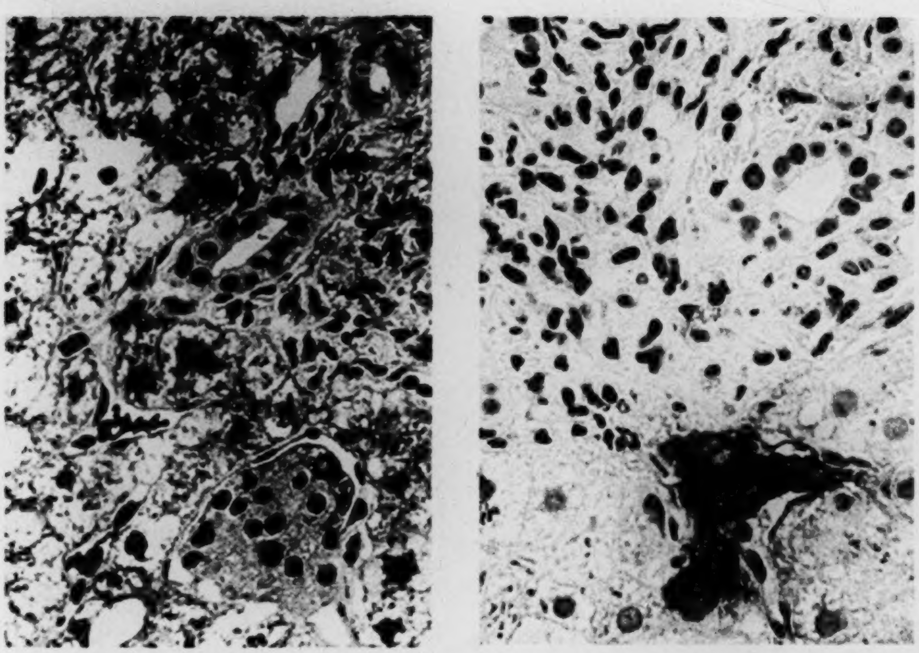


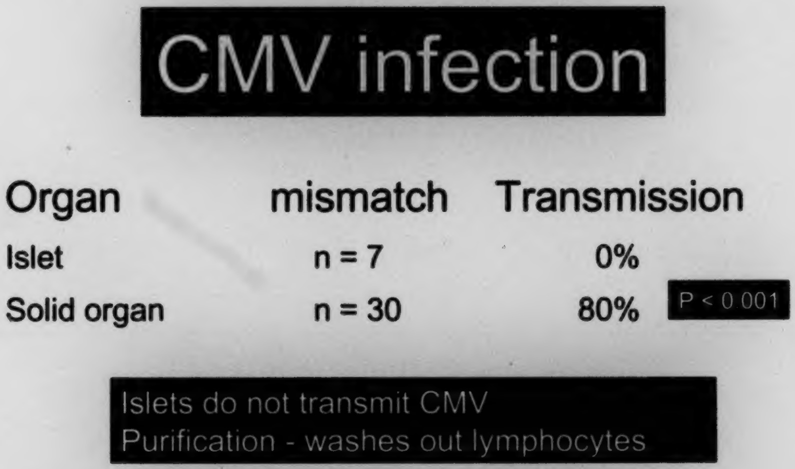
Figure 8.14: Percutaneous single-pass liver biopsy taken at one month after first transplant (18 gauge core) stained with H&E (left) and insulin (right)

(courtesy of Dr Lawrence Jewell).

A percutaneous liver biopsy was obtained in one patient at one month after the first transplant, using an 18-gauge core. The indication for liver biopsy was new appearance of inhomogeneity on ultrasound examination. There were three separate islets identified in this biopsy. One islet is shown above, lying in close proximity to the portal triad, portal vein, hepatic vein and bile duct. Immunohistochemistry staining with insulin antibody confirms beta cells in a contiguous section. There was no evidence of inflammatory infiltration in this biopsy, suggesting that immunosuppression had controlled both autoimmune and allo-immune reactivity. Microvesicular steatosis was identified in the surrounding liver tissue, that may have been a local response to the presence of the islet, or a secondary phenomenon related to drug therapy.

8.7 COMPLICATIONS – POTENTIAL AND OBSERVED

In terms of complications, there have been no episodes of CMV infection (as evidenced by an absence of seroconversion or disease), despite infusion of mismatched preparations (positive donors into negative recipients) in 7/15 cases (p <0.001, Table 8.4). In contrast, the transmission rate in liver and kidney transplants carried out concurrently was 80%. All recipients were given oral ganciclovir for 14 weeks post transplant, irrespective of mismatch status. This data strongly suggests that an islet transplant does not contain a sufficient threshold number of lymphocytes to facilitate CMV transmission. Presumably this difference is a benefit of the islet purification process, as whole pancreas transplants readily transmit CMV infection; this observation requires confirmation by quantitative PCR analysis of impure and purified islet samples. The data also supports a more selective use of oral ganciclovir only in CMV negative recipients receiving CMV positive grafts.



CMV infection

Organ	mismatch	Transmission	
Islet	n = 7	0%	
Solid organ	n = 30	80%	P < 0.001

Islets do not transmit CMV
Purification - washes out lymphocytes

Table 8.4: Absence of CMV transmission from CMV positive donors to CMV negative recipients, compared with an 80% transmission rate in liver and kidney transplantation *(data courtesy of Dr Jutta Preiksaitis)*

A previous concern in islet transplantation was that recipients sensitized to donor antigens might then be unable to be successfully matched for a kidney transplant if they ultimately progressed to renal failure(*8*). We found that none of the recipients were sensitized to donor antigen by prospective specific cross-matching to donor antigen (AHG cross match of recipient serum to donor splenocytes), and all recipients continue to have negative panel reactive antibodies (PRA) in long term follow up. This important finding suggests that the risks of recipient sensitization may be low in islet-alone transplantation under the cover of daclizumab/sirolimus/tacrolimus immunosuppression. This contrasts to previous studies in islet transplantation where recipients were sensitized to donor antigen when treated with cyclosporine/azathioprine immunosuppression(*9*).

The complications from islet transplantation encountered in our series thus far include:

Non-life-threatening bleeds from the hepatic puncture site in 2 cases. This risk was considerably reduced by lowering the dose of systemic heparin given intraportally at the time of islet infusion. We have recently changed the size of catheter used for islet implantation (4Fr Cook O'Kelly islet catheter, 900µm inner luminal diameter), as we believe this will have the most bearing on risk of post-procedural bleeding. We are currently recommending a heparin dose of 35 U/kg delivered into the portal vein, mixed in with the first syringe of islets – since this might reduce islet loss related to thrombotic events occurring at the time of implantation(*2, 10*).

A peripheral branch-vein thrombosis of a right lateral branch of the portal vein in one case. The main portal vein remained patent. We believe this complication was technical in nature, relating to islet infusion into a peripheral branch rather than in the central portal vein, and may have been compounded by use of a liquefied preparation of thrombostatic agent in this case that embolized down the catheter tract on completion of the procedure.

Puncture of the gallbladder with a fine Chiba needle in one case. The patient developed pain related to this, with post-procedural peritonism, but there was no evidence of bile leak on scanning, and this complication resolved without requiring further intervention.

Three patients developed transient elevation in liver function, which resolved over time. **Superficial buccal ulceration** has been a troubling side effect of sirolimus therapy in the majority of recipients in our experience.

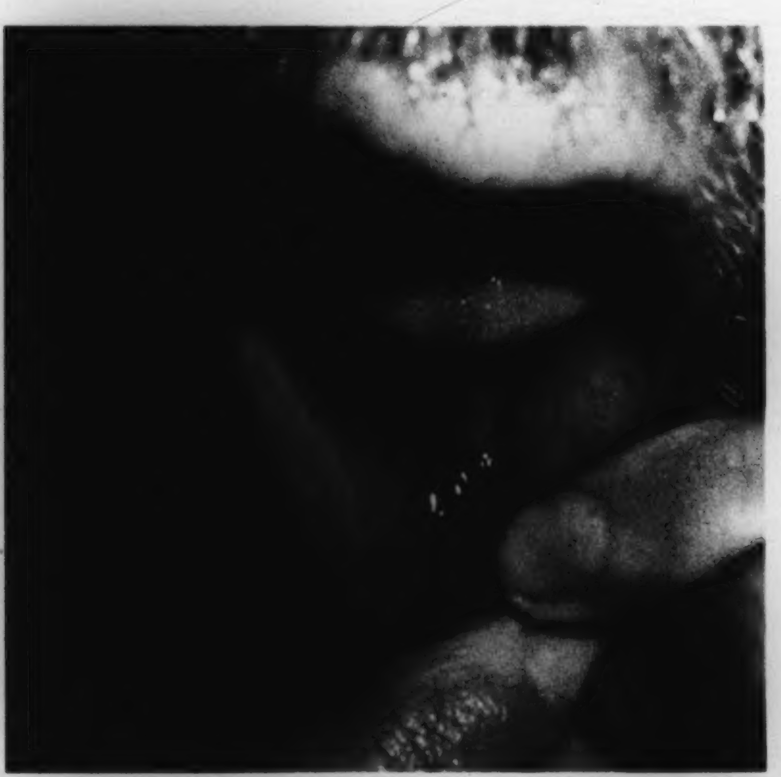


Figure 8.15: Aphthous ulceration seen on the inner lip of an islet recipient – induced by sirolimus

This complication has resolved in all cases over time, and was likely helped by lowering the target sirolimus level and by conversion to the tablet rather than a liquid formulation of this drug. It is unclear why these patients have had such a high incidence of this minor complication.

Sirolimus-related dyslipidemia was encountered a high proportion of patients, resulting in the initiation of statin therapy in 9/15 patients. While this complication raises important concerns regarding long-term risk of accelerated atherosclerosis in patients with underlying diabetic vessels, some recent preliminary observations suggest that this risk may be lower than initially perceived. A paradox is emerging, as sirolimus treatment has been shown to abolish accelerated atherosclerosis in Apo-E knockout mice, and in porcine models of atherosclerosis(*11*). More recently a series of patients underwent coronary artery stent placement with stents coated in sirolimus, and preliminary data suggests that these stents may have *reduced* risk of occlusion over time(*12*).

An additional concern has been a rise in serum creatinine in two patients with pre-existing impairment in renal function from diabetic retinopathy (Figure 8.16). The elevation in creatinine has recently been stabilized by the complete withdrawal of calcineurin inhibitor and replacement with mycophenolate (Cellcept) at a dose of 500mg twice daily. This concern has not been reflected in the group as a whole, where the mean serum creatinine has not changed over time (87.4 ± 7.8 µmol/L pre-transplant vs. 97.0 ± 14 µmol/L in current follow-up, p=NS). Comparison of creatinine clearance data also reveals no significant change comparing pre-transplant with current values (1.72 ± 0.12 mls/1.76m2/min pre-transplant vs. 1.66 ± 0.12 ml/1.76m2/min in currently, p = NS). This observation clearly demonstrates that even renal sparing low-dose tacrolimus / sirolimus therapy may have deleterious effects in circumstances where the baseline nephron mass is marginal.

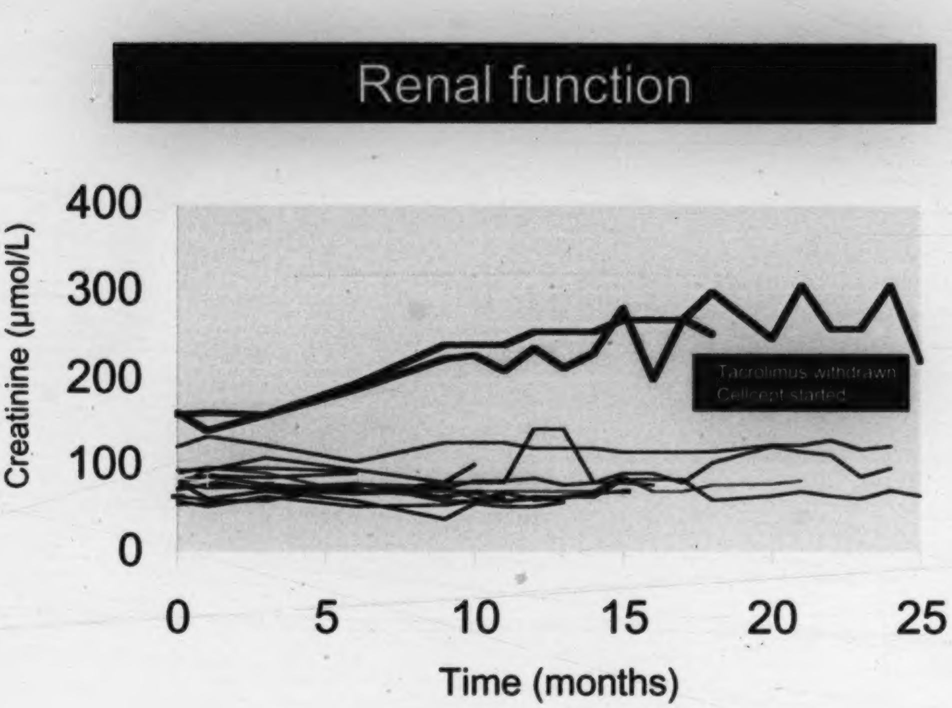


Figure 8.16: Changes in serum creatinine over time, and effect of withdrawal of calcineurin inhibitor.

There have been no life-threatening complications observed to date, and in particular there have been no cases of post transplant lymphoproliferative disorder (PTLD), malignancy or severe infections observed. It is recognized that the potent immunosuppression required to protect islet grafts from rejection and autoimmune recurrence carries these risks. The precise risk of lymphoma is not known with the current protocol, but may be estimated to be of the order of 1-2%, based on other clinical studies in patients receiving anti-IL2R mAb therapies(*13*).

8.8 QUALITY OF LIFE STUDIES

Quality of life

Waiting list
Islet recipients
Pancreas alone

It is anticipated that with marked improvement in glycemic control with elimination of hypoglycemic events in patients undergoing islet transplantation, the patients' quality of life should improve. The avoidance of major surgery, with the transplant procedure and follow-up largely outpatient based, this therapy has been particularly attractive for the patient. Trying to quantify a less tangible benefit of islet transplantation in terms of impact on quality of life, collaboration has been built with Dr Jeffrey Johnson (Institute of Health Economics, University of Alberta). Dr Sulaiman Nanji, has been instrumental in helping to coordinate an initial pilot study, comparing waiting list patients matched for age, gender and indication with islet recipients post transplant. A further collaboration with the University of Memphis (Dr Osama Gaber, Rebecca Winsett, Dr Donna Hathaway) has further allowed us to compare outcomes in patients undergoing pancreas-alone transplantation for similar indications.

The preliminary study has compared a standard validated general quality of life tool (SF-36) and Health Utility Index score (HUI), and ongoing studies will further utilize a validated immunosuppression complication scoring system and a specific hypoglycemia related score in a more detailed prospective study planned as an adjunct to the Immune Tolerance Network islet multicentre trial.

While initial trends have suggested that islet transplantation may be beneficial, and possibly superior to whole pancreas transplantation (Figure 8.17), more detailed prospective, controlled studies are now justified to determine significance of these preliminary findings.

Outcomes from a well-conducted prospective quality of life study may further contribute to the ongoing debate of whether some patients might benefit from receiving a 'sub-therapeutic' single-donor islet preparation with a primary goal of stabilizing glycemic control rather than attainment of insulin independence(*14*, *15*). This issue is likely to take on more relevance once tolerance can be successfully achieved in the clinic, and where the up front procedural and inductive risks of attaining this goal are so low that the added trade-off of insulin independence may no longer be mandated. Until this time, insulin independence should remain the dominant force to ensure future success in the field(*14*).

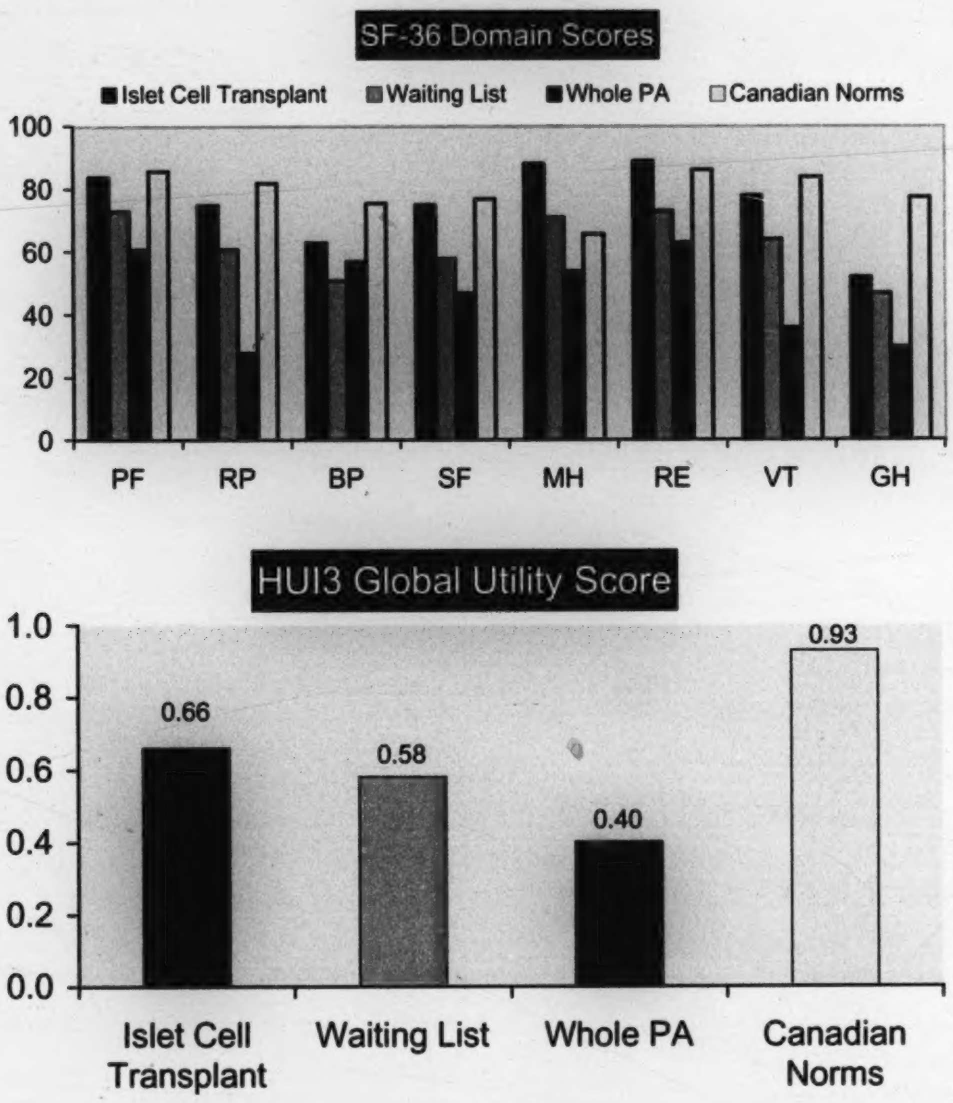


Figure 8.17: Preliminary data – Quality of life in islet recipients compared to waiting list patients or whole pancreas recipients: SF-36 domain scores and Health Utility Index

(Collaboration with Dr Jeffrey Johnson, Dr Sul Nanji, and Dr Osama Gaber et al)

8.8 COLLABORATIONS, INTERNATIONAL MULTICENTRE TRIAL OF THE EDMONTON PROTOCOL AND THE IMMUNE TOLERANCE NETWORK

It is evident from the above data that the "Edmonton Protocol," while not perfect, has led to an unprecedented improvement in clinical outcomes of islet transplantation for autoimmune diabetes in a limited number of recipients at a single centre. This success has highlighted some key challenges that would be best tackled by strong collaborative interactions between a number of international institutions. To help move this collaboration forward, it was felt that a strong baseline level of success using a common initial protocol would provide an opportunity to compare future protocol refinements in a controlled fashion. The Immune Tolerance Network (funded jointly by the National Institutes of Health and Juvenile Diabetes Foundation) elected to invest in this opportunity, recognizing the future potential of islet transplantation in development of novel tolerance protocols for the control of both autoimmunity and allo-transplantation. The availability of islet transplantation as a clinical research tool provides a unique opportunity to evaluate novel therapies where uncertainty regarding efficacy would be unlikely to lead to disastrous clinical consequence – if the therapy is safe but ineffective, the patient may simply return to insulin therapy rather than face the disastrous consequences of losing a life-sustaining graft.

The first international multicentre trial in clinical islet transplantation will replicate the "Edmonton Protocol" in a total of 40 patients across a total of 10 university centers, with 7 in North America (Edmonton, Miami, Minneapolis, National Institutes of Health, Seattle, St Louis and Harvard) and 3 European sites (Giessen, Milan and Geneva).

The initial implementation of this trial has been a major challenge because of requirements by the Federal Drug Agency (FDA) to standardize islet processing and final product quality control testing .As a result of this, standardized equipment has had to be supplied from a single source to each centre, and rigorous qualifying sample data has been required to confirm a centre's suitability to proceed with the trial. Initial progress was slowed by inconsistencies in the preparation of a single batch of the low-endotoxin collagenase, Liberase^{TM}. This challenge has recently been solved through the collaborative exchange of isolation data and expertise in interpreting patterns of success at the co-Principal Investigators' institutions. The current optimal collagenase blend will target a Neutral Protease activity of 62,000 units and Wünch activity of 2,200 units. Elimination of the lyophilization stage has allowed the manufacturers to increase their lot size substantially, reducing the requirement for multiple sub-lot testing at multiple sites.

While the immunosuppressant protocols may be readily reproducible across different international sites, controlling for inconsistency in islet quality in centers with limited experience in islet isolation may prove to be a major limiting factor in this trial.

Encouraging preliminary data from the Universities of Miami, Minneapolis, the National Institutes of Health and at Northwestern University in Chicago have already validated that the "Edmonton Protocol" results are reproducible at external sites, and that insulin independence is readily achieved. This bodes well for the international trial, and for the ultimate transition from whole pancreas transplantation to islet transplantation.

8.9 RESEARCH CHALLENGES AHEAD

Major research challenges remains in terms of improving the functional survival of engrafted islets – and coordinated trials in the near future will be designed to address: a) newer drug therapies without diabetogenic side effects (calcineurin inhibitor-free) (*13*), b) anti-inflammatory strategies (anti-TNF alpha monoclonal therapy, anti-platelet drugs, complement inhibitors) and c) improved methods for islet isolation and in vitro expansion. Some of these issues will be further discussed in Chapter 9.

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CHAPTER 9:

CONCLUSIONS, SUMMARY AND FUTURE TRENDS IN THE EVOLUTION OF CLINICAL ISLET TRANSPLANTATION IN THE CURATIVE TREATMENT OF DIABETES MELLITUS

9.1 OVERVIEW

The recent dramatic transformation in outcome of clinical islet transplantation reported by our group has secured a future for this therapy in diabetes(*1-3*). The Islet Transplant Registry had previously established a one-year insulin independence rate of less than 10% in 447 previous transplant attempts in the previous decade under cyclosporine and steroid-based immunosuppression(*4, 5*). Using a novel steroid-free combination of daclizumab, sirolimus and low-dose tacrolimus, designed to prevent both autoimmune recurrence and allograft rejection while avoiding diabetogenic toxicity from highly concentrated drug delivery in the liver (the site of islet implantation), the one-year rate of insulin independence rose to 100% in 7 patients receiving islet-alone grafts. The "Edmonton Protocol" further optimized islet function by immediate graft processing, controlled delivery of a purified low-endotoxin collagenase enzyme and transplantation to limit cold ischemia, avoided culture and exposure to xenoproteins (fetal calf serum), and a double transplant ensured a total average of 830,000 islets (9,000 to 10,000 islet equivalents per kg recipient body weight) into the liver via the portal vein. The protocol was designed to address a series of barriers that had limited success previously, as defined by Hering(*6*).

More recently the Edmonton series has been expanded to 16 patients. All patients have evidence of ongoing graft function, and all but four remain completely insulin free currently. Four patients remain insulin free beyond two years of transplantation. These patients have approximately one fifth of normal insulin reserve, with no loss of mean function over time. The novel immunosuppressant regimen prevented sensitization to donor antigens, as shown by a negative panel reactive antibody (PRA) in all cases; this was of potential concern previously to patients who might one day require matching for renal transplantation(*7*).

While there is no proof as yet that successful islet transplantation will prevent secondary diabetic complications in humans, maintenance of normal glycosylated hemoglobin and complete correction of diurnal glucose swings without graft deterioration over time provide compelling implications that islet transplantation will be at least as effective as whole pancreas transplantation in controlling and reversing early diabetic complications(*8-10*)

Will islet graft function be maintained in the long-term, or will patients require supplemental islet preparations over time? The evidence is optimistic, as recent studies indicate that islet autograft and allograft function can be preserved for as long as 13 years after transplantation(*11, 12*). Advances in anti-rejection treatments that virtually eliminate graft loss from acute or chronic rejection may eliminate the potential for islet degradation over time, and the anti-inflammatory effects of sirolimus may further promote islet survival in an intrahepatic environment(*3, 13*). However longer follow-up is needed in larger numbers of patients before we can be certain that recurrent autoimmunity will not lead to graft degradation even in the face of systemic immunosuppression(*14*). Long-term exposure to high-dose tacrolimus leads to islet structural damage over time; it remains to be seen whether low-dose tacrolimus will have a less damaging effect(*15*).

Now that islet transplantation has clearly been shown to work with a high degree of success in a limited number of patients with type 1 diabetes, some major challenges lie ahead (Figure 9.1). Only by overcoming these hurdles can islet transplantation become the ultimate 'cure' for the 130 million patients with diabetes worldwide(*16*).

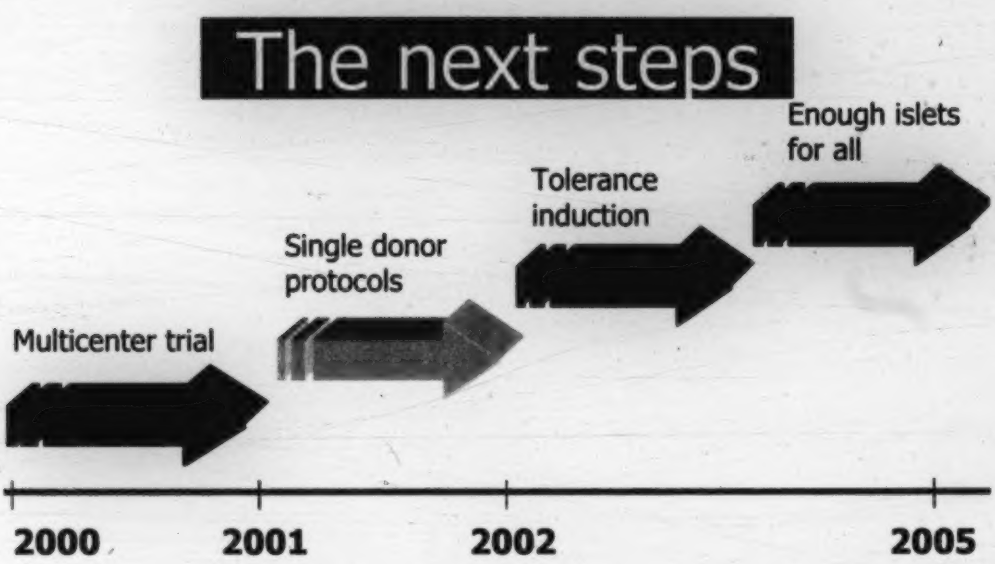

The next steps
Enough islets for all
Tolerance induction
Single donor protocols
Multicenter trial
2000
2001
2002
2005

Figure 9.1: Current and future challenges that, if solved, will increase the applicability of islet transplantation (or alternative cellular replacement therapies) towards a cure for diabetes.

The Immune Tolerance Network's initial trial, funded jointly by the National Institutes of Health and Juvenile Diabetes Foundation, will replicate the "Edmonton Protocol" in a total of 40 patients across 10 centers in North America and Europe. Preliminary results in four separate US institutions have already confirmed the successful reproducibility of this protocol. While the immunosuppressant protocols may be readily reproducible across the world, controlling for inconsistency in islet quality in centers with limited experience in islet isolation is likely to prove to be a major limiting factor. A major concern will be how many optimal pancreas donors will be 'consumed' during the steep learning curve as a plethora of new islet isolation centres attempt to master the techniques; an estimate of over 50 new sites are predicted to open in the next twelve months.

9.2 SINGLE DONOR ISLET TRANSPLANTATION

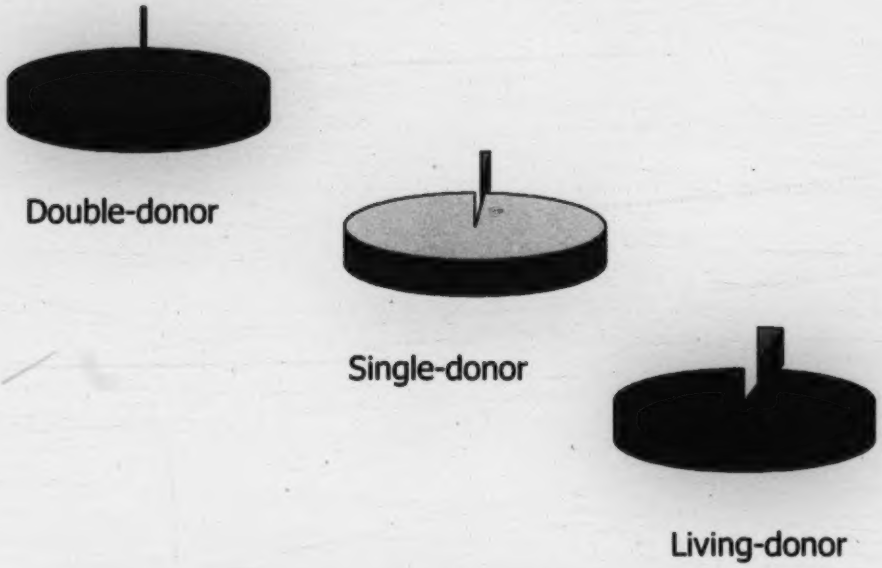


Figure 9.2: Advances in "single donor" and possibly living donor islet transplants, while of importance to the field, will only have very modest impact in treating vast potential pool of 130 million patients with diabetes worldwide.

(Adapted from Hering and Ricordi, 2001)

The next challenge will be to obtain consistent success with a single rather than a double-donor transplant. Advances in islet isolation techniques, taming of endogenous pancreatic enzyme activity during digestion phase of islet isolation, coupled with insulin sensitizing drugs and anti-inflammatory strategies given to the recipient (such as anti-TNF alpha, soluble complement receptor antibodies or antioxidant therapies) may help to limit non-immune mediated immediate graft loss after implantation(*17, 18*). One day it might be possible to treat three recipients from a single donor, based on experimental studies with non-purified islet grafts(*19*). If excellent outcomes of islet transplantation are reproduced across multiple centers worldwide, and success is maintained long-term using single donor islet preparations, this will accelerate the transition of islet transplantation as the standard of care for many more patients with diabetes, possibly ultimately relegating the whole pancreas transplant procedure to the history books.

While consistent success of single-donor protocols would represent a significant advance to the science and the field, it will have only minimal impact in increasing access to islet transplantation for the 130 million people with diabetes worldwide (Figure 9.2). A clear advantage however would be for collaborating centres to complete twice as many successful transplants in the same time-frame – thereby accelerating the pace of research trials.

A rapid expansion in level of clinical islet transplant activity is critically dependent upon support in a variety of key areas currently, and every step is vital to the overall success of the endeavor. A limited cadaveric organ donor pool will always remain a focus of paramount importance, but, contrary to popular belief, is not currently the major rate-limiting step in preventing rapid expansion of islet transplantation activity. Rather, the practical process of guaranteeing that every available cadaveric pancreas is procured for islet isolation wherever possible must now become a priority goal. For instance, in the year 2000 in Canada there were 473 organ donors; only 65 of these organs were used for whole pancreas transplantation. Two thirds of these organs were never recovered from suitable donors, and a significant proportion arrived at the islet isolation laboratory beyond eight hours after procurement. The situation is different in the US, where a much larger number of whole pancreas transplants are performed on an annual basis.

Meticulous care in the surgery of pancreas procurement is critical, with the pancreas removed with minimal handling to maintain the pancreatic capsule in an intact state. Maintenance of the core pancreatic temperature between +4 - +10°C while other organs are being removed in the multiorgan retrieval is critical to the optimal yield and functional viability of the procured islets. Strong cooperation between organ procurement organization, procuring surgeons, and the isolation laboratory remain essential, and adequate access to covering funds may be key to ensuring that all available cadaveric pancreata are referred expeditiously. For the future success of islet transplantation it will be necessary in some circumstances to give priority to pancreas procurement for islets rather than whole pancreas transplantation, with the rational being that a pancreas destined for islet isolation has a more critical cold ischemic window (ideally a minimum of 8 hours) than its solid organ counterpart (that may occasionally tolerate up to 30 hours of cold ischemia). Prolonged cold ischemia beyond 8 hours frequently leads to confounding effects of endogenous exocrine enzyme activation that may critically interfere with the isolation process and therefore the ultimate success in terms of optimal yield and functional viability. Access to rapid jet transportation to minimize ischemia is predicted to enhance successful islet isolation thereby translating to a marked increase in clinical islet transplant activity.

Strategies offering most promise in facilitating single-donor islet transplantation include:

a) **Anti-inflammatory treatments** to optimize islet engraftment – including anti-TNF alpha monoclonal antibody sequestration therapies, aspirin and other more powerful platelet antagonist drugs, prolonged course outpatient low molecular weight heparins, and soluble complement receptor-1 antagonists (e.g. TP-10). The drug deoxyspergualin is an intriguing compound with a unique mechanism of action that might facilitate islet engraftment in addition to minimizing the risk of rejection (refer to Chapter 2).

b) **Calcineurin-inhibitor free immunosuppression** – an approach that may be facilitated by combination of Cellcept with sirolimus, but this combination may not offer maximal immunologic efficacy and may lead to increased side effects through overlap in risk of neutropenia that might be dose limiting in some cases. More potent therapies with increased specificity may offer promise since novel pathways may be explored and controlled – e.g. FTY720 combined with rapamycin analogues (see Chapter 2), but these therapies will mandate an adequate safety record in Phase I/II trials before justifying their use in a diabetic patient where the goal may be to exchange insulin for safe immunosuppression. Calcineurin-inhibitor avoidance therapies will have the added advantage of helping to preserve renal function where there is underlying diabetic nephropathy before transplantation(*20*).

c) **Anti-adhesion therapies** – based on anti-CD11a treatment (anti-LFA1, see Chapter 2), also offer a rational approach to calcineurin inhibitor avoidance, and are likely to be tested clinically in combination with sirolimus in initial studies in islet transplantation.

d) **Campath-1H** – anti-CD52 therapy has already met with success in preliminary studies in clinical renal transplantation, and by combination with sirolimus and complete avoidance of calcineurin inhibitors offer the potential both the promote metabolic function of islet grafts, minimize immunologic attack, or even promote induction of a tolerant or near-tolerant state. This antibody is being intensively studies for this potential at the present time, and will shortly be evaluated in clinical islet transplantation.

9.3 MINIMAL IMMUNOSUPPRESSION OR TOLERANCE – HOW CLOSE TO THE CLINIC?

The possibility of achieving a permanent state of unresponsiveness (tolerance) to an allograft without the need for chronic immunosuppression remains an important focus in transplantation research. However attainment of a tolerant state is not the only presiding factor limiting the rapid, broader application of islet transplantation in the earliest stages of diabetes, including children. If the risk of chronic long-term immunosuppression could be substantially reduced by a dramatic reduction in degree of systemic immunosuppression, this would accelerate progress towards the ultimate goal. Islet transplantation may prove to be a challenging model to establish tolerance because the dual forces of autoimmune and alloimmune reactivity must both be neutralized, and different mechanistic approaches may ultimately be required to achieve this. New approaches will therefore be sought to minimize, but that perhaps may not eliminate dependence on very low dose immunosuppression as a practical means to equalize the risk-benefit ratio in patients with diabetes that wish to exchange insulin for an islet transplant.

Some new approaches will be designed to take advantage of the normal **graft accommodation** response – a process that is incompletely understood mechanistically, but permits dramatic tapering of immunosuppressive dose without graft destabilization after an extended period, and is seen in all clinical transplant situations. If this accommodation response could be accelerated, single-agent low-dose maintenance immunosuppression could be given in circumstances that would otherwise have been sub-therapeutic. The potential risk of lymphoma, other malignancies and drug-related side effects would then be substantially reduced.

The administration of novel compounds or selected mAb therapies given at the time of islet implantation may interference with alloimmune activation pathways thereby accelerating the accommodation response, ultimately permitting sub-therapeutic low-dose maintenance sirolimus monotherapy to control autoimmunity and prevent rejection. The most promising therapies to explore this minimal immunosuppression approach, and anticipated for application shortly in clinical islet transplant pilot trials include: i) **Campath-1H** (together with infliximab therapy to reduce cytokine storm and promote islet engraftment), ii) **non-mitogenic anti-CD3 (hOKT3γ_1-ala-ala)**, and when clinically available, iii) **LEA29Y** or iv) **anti-CD45RB** (refer to Chapter 2).

The ultimate challenge will be to carry out successful transplantation without subjecting the recipient to lifelong increased risk of malignancy and infection. Tolerance strategies, the long sought-after 'Holy Grail' of transplantation, will likely be developed first in islet transplantation. The consequence of failure of efficacy of a tolerance treatment would simply result in a patient's return to insulin, in contrast to the potential loss of a life-sustaining graft such as a heart or liver transplant that could precipitate patient demise. Exciting progress in the development of a variety of co-stimulatory blocking antibodies that prevent 'Signal 2' activation while leaving 'Signal 1' T-cell receptor antigen engagement unaltered, have shown initial promise in large animal primate models, but further testing of one potent anti-CD40 ligand blocking antibody (Hu5C8) has been halted due to unexpected thromboembolic complications in early clinical trials in rheumatoid arthritis that culminated in a patient death(*21-23*). Kenyon *et al* did not observe these complications in their nonhuman primate model, but clearly showed that monthly maintenance anti-CD154 therapy extended functional islet allograft survival beyond one year; this antibody was also effective in reversal of early acute rejection episodes(*21*). Safety testing of these novel approaches has to be of paramount importance if applied to islet transplantation, as the risk-benefit ratio must reflect the fact that the underlying diabetic condition is not immediately life threatening. If techniques to induce tolerance to allo-antigens prove to be inadequate, or if grafts are destabilized by triggering events such as an acute viral illness, a reasonable compromise may be to use a costimulatory blocking or bone-marrow conditioning adjuvant strategy in concert with very low-dose immunosuppression to diminish the risk of malignancy and infection to almost zero. Sirolimus monotherapy at low dose would be one obvious choice in this setting, since priming of activation induced cell death remains unimpaired in activated T lymphocytes, and is therefore 'tolerance-compatible'(*24-27*). Glucocorticoid treatment may also interfere with active tolerance pathways(*28, 29*). Controversy persists in terms of how essential it will be to eliminate calcineurin inhibitor therapy in tolerance regimens, as a small number of patients have achieved tolerance to kidney allografts following donor bone marrow transplantation from living donors under the temporary cover of cyclosporine therapy(*30*). This 'mixed chimerism' strategy represents one of the few strategies to have been successful clinically in induction of stable tolerance. Exploration of strategies aimed at promoting mixed chimerism and tolerance in clinical liver and kidney transplantation through infusion of donor enriched CD34-positive stem cells have led to improved long term graft outcomes in kidney and liver transplantation, possibly through impact in prevention of chronic rejection(*31-34*). Ricordi *et al* are currently exploring the potential of cryopreserved donor CD34-enriched stem cell fractions to promote islet allograft acceptance with immunosuppressive withdrawal after one year, and definitive results are eagerly awaited.

It remains to be seen whether strategies that provide robust tolerance to alloantigens will also effectively control recurrence of autoimmunity in patients with diabetes. Experimentally, techniques to induce either central or peripheral tolerance have shown benefit, but the most promising approaches have used a combined approach to achieve mixed chimerism. The combination of total body irradiation with bone marrow transplantation and two doses of anti-CD40L antibody was able to induce donor-specific allotolerance without recurrence of autoimmunity with prolonged islet graft survival in overtly diabetic NOD mice(*35*). Graft function was maintained beyond 100 days with robust tolerance to donor-strain skin grafts in this model(*35*).

One exciting strategy that has shown considerable promise in two large animal primate studies is tolerance induced by an anti-CD3 based diphtheria-conjugated T-cell immunotoxin ($F(Ab)_2$ – immunotoxin). Thomas *et al* have recently shown that when combined with an inductive course of deoxyspergualin, streptozotocin-diabetic and spontaneously diabetic primates became operationally tolerant to islet allografts; insulin independence was maintained beyond one year in four of seven animals(*36*). The group had previously shown that renal allografts transplanted into a similar nonhuman primate model under cover of two weeks of inductive diphtheria immunotoxin were able to achieve rejection-free tolerance for over four years without need for maintenance immunosuppression(*37*). The underlying mechanism is proposed to involve immunotoxin-mediated depletion of circulating and sessile T-cells of both naïve and memory sub-types; the addition of deoxyspergualin may have concomitantly blocked activation of pro-inflammatory cytokines, led to cytokine deviation towards a TH-2 phenotype, and furthermore interfered with dendritic cell maturation(*36*).

9.4 WILL ISLET TRANSPLANTATION PLAY A ROLE IN TYPE 2 DIABETES?

Will islet transplantation be effective in type 2 diabetes? The answer is uncertain at present, but based on increased peripheral insulin resistance, it is possible that up to ten times as many islets might be required to sustain insulin independence. Preliminary testing in liver transplant recipients suggests that there may be some benefit(*38*). Certainly strong evidence is mounting that tighter metabolic control will delay progression of secondary diabetic complications even in type 2 diabetes(*39, 40*).

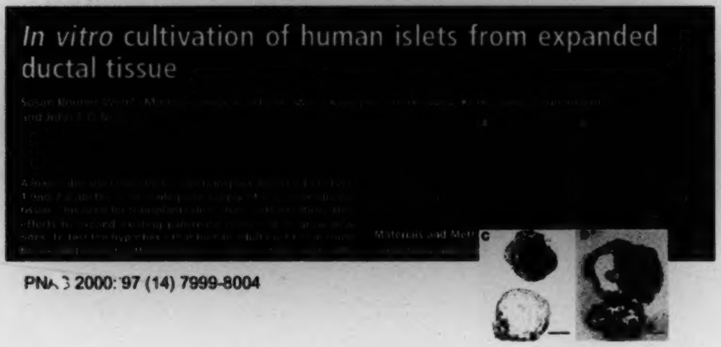
9.5 ALTERNATIVE TISSUES SOURCES

The final challenge therefore will be to find an alternative tissue source to provide sufficient insulin producing, glucose responsive cells to treat all patients with diabetes worldwide. As illustrated in Figure 9.2 (above), clinical islet transplantation will never be able to fulfill its potential as a cure for diabetes if activity remains restricted entirely to islets derived from a limited cadaveric organ donor pool. The fact that diabetes touched people of all walks of life, being the third commonest disease and fourth leading cause of death in the Western world, will hopefully heighten awareness and lead to a dramatic increase in organ donation amongst non-diabetic relatives. It is estimated that less than 0.5% of patients with Type 1 diabetes could benefit from islet transplantation presently, and proportion will not increase significantly even if single-donor transplantation becomes uniformly successful. If islet transplantation for type 2 diabetes becomes a reality, the task of finding the cure for all diabetics through cadaveric islet transplantation will become futile. Alternative sources of insulin-secreting tissues are now being investigated intensively to fill this niche.

9.5.1 LIVING DONOR ISLET TRANSPLANTATION – FUTURE POTENTIAL

A series of over 50 living donor segmental pancreas transplants have been completed at the University of Minnesota(*41-43*). Initial developmental experience suggested a modest increased donor risk of procedural complications, impaired glucose tolerance or more seriously, new diabetes induction in healthy donors followed long-term(*44*). More recently, careful selection to avoid obese donors, those with pre-resectional impairment of glucose tolerance or those at increased risk of diabetes due to positive serological autoimmune antibody markers (ICA, GAD or mIAA) has largely eliminated this risk. Furthermore, recent developments in surgical technique including the potential for laparoscopic or hand-assisted retrieval, may enhance the palatability from a donor's perspective, provided technical complications such as pancreatic fistula are avoided. The natural extension of this technique would be to carry out islet transplantation from living donors, since the potential risk to the recipient should be considerably less than a segmental pancreas transplant. The challenge will be to deliver an adequate islet engraftment mass to secure insulin independence with the technique, since recipients of the Edmonton Protocol have typically required two or more entire donor pancreata to achieve a satisfactory metabolic result. One potential may be to consider infusion of unpurified or partially purified islet preparations, since this was the traditional technique used previously in successful human islet autografted patients after total pancreatectomy for chronic pancreatitis. Early experimental studies in large animals suggested that up to three recipients might be successfully cured by unpurified islet grafts prepared from a single donor(*19*).

“Islet farming”



In vitro cultivation of human islets from expanded ductal tissue

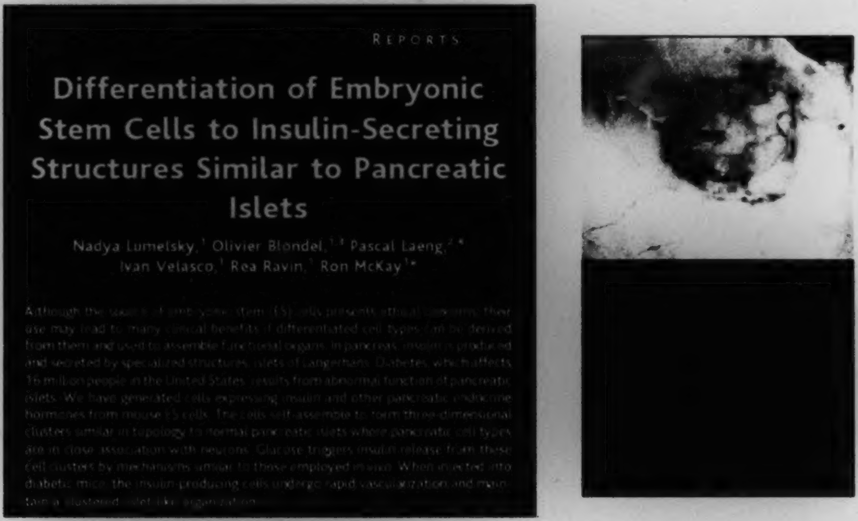
PNAS 2000: 97 (14) 7999-8004

Figure 9.3: Islet neogenesis from ductal stem cells (Bonner-Weir et al)

9.5.2 ISLET NEOGENESIS, POTENTIAL OF STEM CELLS OR XENOGENEIC ALTERNATIVE SOURCES

While living donor islet transplantation offers a unique potential as an alternative source of human islets, the approach will likely remain controversial so long as a healthy donor is placed at potential risk from procedural complications. Intensive research in ‘islet farming’ may provide one answer, where new islets are grown from pancreatic ductal elements that would have otherwise been discarded during the islet purification process (Figure 9.3) (*45*). The success of this approach related to the provision of a stabilizing extracellular matrix (Matrigel) together with islet growth factors. While promising, it is estimated that only 30,000 new islets could be derived from each human pancreas preparation using this approach, which clearly severely hampers its potential applicability in its current stage of development.

Alternative solutions include a search for the elusive pancreatic islet stem cell(*46, 47*), use of islet neogenesis-promoting peptides such as INGAP(*48*), expansion of cloned human insulin-producing cell lines(*49*), or through tissue engineering of non-beta cells to secrete insulin(*50*).



REPORTS

Differentiation of Embryonic Stem Cells to Insulin-Secreting Structures Similar to Pancreatic Islets

Nadya Lumelsky,[1] Olivier Blondel,[1,3] Pascal Laeng,[2,*] Ivan Velasco,[1] Rea Ravin,[1] Ron McKay[1,*]

Although the source of embryonic stem (ES) cells presents ethical concerns, their use may lead to many clinical benefits if differentiated cell types can be derived from them and used to assemble functional organs. In pancreas, insulin is produced and secreted by specialized structures, islets of Langerhans. Diabetes, which affects 16 million people in the United States, results from abnormal function of pancreatic islets. We have generated cells expressing insulin and other pancreatic endocrine hormones from mouse ES cells. The cells self-assemble to form three-dimensional clusters similar in topology to normal pancreatic islets where pancreatic cell types are in close association with neurons. Glucose triggers insulin release from these cell clusters by mechanisms similar to those employed in vivo. When injected into diabetic mice, the insulin-producing cells undergo rapid vascularization and maintain a clustered, islet-like organization.

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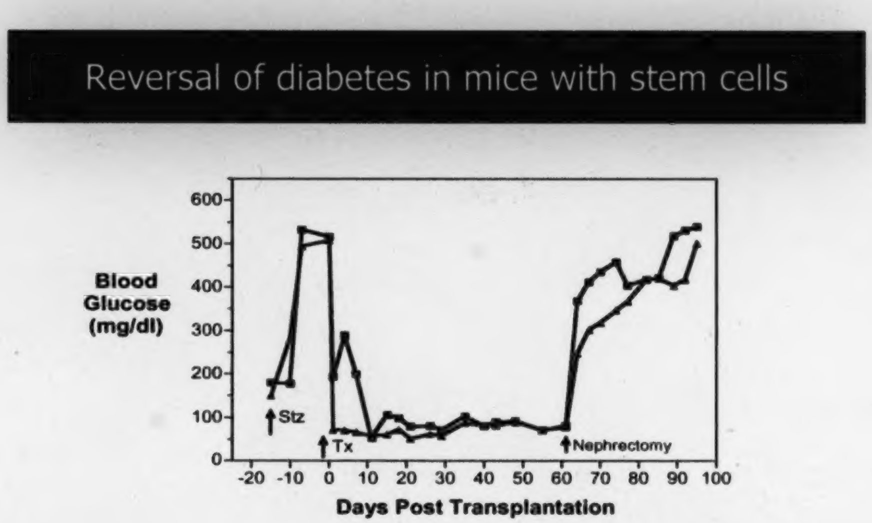
Figure 9.4: Differentiation of embryonic stem cells into islet-like clusters, with capacity for revascularization (Lumelsky *et al*, Science 2001)

A recent report by Lumelsky *et al* demonstrated that mouse embryonic nestin-positive stem cells could be coaxed into differentiating into three-dimensional clusters similar to islets, and could secrete insulin in response to elevated glucose (Figure 9.4) (*51*). After transplantation the cell clusters revascularized, but were unable to fully correct hyperglycemia in diabetic mice – possibly as a result of relatively low-level expression of PDX-1(*51*). Skorechi *et al* (Haifa, Israel, unpublished data) have had similar success in inducing human embryonic stem cells to secrete insulin – in these studies, the inner cell mass of the 64 cell stage human blastocyst was exposed to high glucose concentrations (25mmol/L), and insulin-positive staining elements were identified subsequently.

Genetic engineering of hepatocytes to secrete a single-chain insulin analogue using an adenoviral construct under control of the L-pyruvate kinase promoter, was able to correct diabetes in mice for longer than 8 months(*52*). Transgenic mice with transformation of intestinal mucosal K-cells were able to secrete insulin in physiological response to hyperglycemia(*53*), and reversal of diabetes in mice with transplantation of embryonic stem cells (Figure 9.5) (*54*) all provide strong conclusive evidence that alternate sources are not far away.



Reversal of diabetes in mice with stem cells

Soria et al, 2000

Figure 9.5: Embryonic stem cell transplantation reverses diabetes in mice (Soria *et al*, Diabetes 2000)

Enthusiasm for xenogeneic tissue sources for islet transplantation has waned recently as a result of concerns regarding zoonotic viral transmission of pig endogenous retroviruses (PERV). The fear of a PERV pandemic of similar magnitude to AIDS has likely been overestimated, but concerns have been fueled by recent reports demonstrating that PERV is transcriptionally active and infectious across species in vivo following transplantation of pig tissues(*55-57*). Transgenic pigs expressing human complement-regulatory proteins have been developed to overcome immediate destructive pathways, but an unacceptable degree of potent immunosuppression is still required (cyclophosphamide) to overcome accelerated acute and chronic rejection, limiting clinical applicability for the present, further dampening enthusiasm for this approach(*58*).

Prospects for the broader application of islet transplantation in type 2 diabetes must await developments in alternate tissue sources(*59*). Perhaps ten times more cells may need to be transplanted in type 2 diabetes to overcome the effects of peripheral insulin resistance.

9.6 SUMMARY AND CONCLUSIONS

The Edmonton series clearly indicates that sustained insulin independence may be achieved in 85% of recipients over time, with effective control of both acute rejection and recurrence of autoimmunity when potent immunosuppression is given. Excellent glycemic control with improvement and normalization of glycated hemoglobin is achievable in most cases, and the risks associated with treatment are much lower than the alternative strategy of whole pancreas transplantation.

Based on the procedural and longer term risks defined in this thesis, it is appropriate to limit treatment to patients that are failing on optimized insulin therapy, and this will likely remain the case until successful tolerance or near-tolerance protocols are developed for safe application in the clinic.

What is certain is that islet transplantation is here to stay, and the recent transformation in outcome will catapult new research efforts that will culminate in a permanent cure for all patients with diabetes.

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